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Clinical and Experimental

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The Journal of Laboratory and Clinical Medicine

VOL. 43 MAY, 1954 NO. 5

CLINICAL AND EXPERIMENTAL

PULMONARY HISTOPLASMOSIS IN A FARM FAMILY: A THREE-YEAR FOLLOW-UP

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CHICAGO, ILL., AND L. W. COMBS, M.D., LOWELL, IND.

INTRODUCTION

SINCE the early part of October, 1950, observations have been made on a farm family (E.) of which six members had pulmonary histoplasmosis of varying degrees of severity. Three had clinical illnesses and extensive involvement of the lungs. The other three had lesser pulmonary findings and no apparent symptoms. The *Histoplasma capsulatum* fungus was isolated from the sputum and bone marrow of the father. The father and his two sons contracted their infections in all probability as a result of inhaling contaminated dust while cleaning an unused silo on their farm. Subsequent studies readily revealed the presence of histoplasma organisms in the soil from the silo. The histoplasma infections in the E. family are of interest in that they illustrate a wide range in clinical and laboratory findings.

An account of the epidemiological aspects and some of the early clinical and laboratory findings of pulmonary histoplasmosis in the E. family have been reported.[1, 2] Likewise, an account of the isolation of the fungus from the silo soil has been published.[3] The purpose of the present report is to give a more detailed account of changing clinical, roentgenologic, and laboratory findings in the different members of the family during the past three years.

CLINICAL AND LABORATORY STUDIES

Eight individuals associated directly or indirectly with the E. farm were included in the study. They were the parents (A. E., Sr., aged 49, S. E., aged 42), three sons (J. E., aged 24, E. E., aged 21, and A. E., Jr., aged 5), two

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daughters (Al. E., aged 19, and M. E., aged 17), and a daughter-in-law (D. E., aged 24). J. E. and his wife, D. E., did not live on the farm but visited there frequently and thus were included in the survey. Previous to the onset of illnesses of the father and his two sons, the members of the E. family had been in good health. E. E. had recently returned from military service.

Clinical Illnesses.—The father and his two sons, E. E. and A. E., Jr., developed acute febrile illnesses at approximately the same time, the last week in September, 1950. They were seen shortly thereafter by one of us (L. W. C.). Their symptoms were fever, chills, nonproductive cough, sweating, weakness, and anorexia. They were treated at home for several days with penicillin and sulfadiazine without abatement of symptoms. On October 9 and 10, x-rays of the chests were taken at St. Mary's Mercy Hospital, Gary, Indiana, and all three were found to have widespread noncalcified pulmonary infiltrates.

TABLE I. SYMPTOMS AND FINDINGS OF THE FATHER AND HIS TWO SONS DURING HOSPITALIZATION AT MERCY HOSPITAL, OCTOBER, 1950

INITIALS AGE (YR.)	E. E. 21	A. E., JR. 5	A. E., SR. 49
Date of admission	October 9	October 10	October 25
Duration of stay (days)	16	6	3
Day of illness on adm.	13	14	27
Symptoms			
Fever	++	++	+++
Cough	++	++	+++
Weakness	++	++	+++
Sweating	++	+	+++
Anorexia	+++	+++	+++
Diarrhea	+	+	+++
Physical	Acutely ill	Mildly ill	Gravely ill
Weight loss (lb.)	10	10	20
Chest râles	++	+	+++
Hepatomegaly	·	–	++
Splenomegaly	–	–	++
Thrombophlebitis	–	–	Left leg
Laboratory			
Chest x-ray	Disseminated noncalcified infiltrates	Disseminated noncalcified infiltrates	Disseminated noncalcified infiltrates
WBC cu. ml.	15,600	11,600	9,600
Polys. per cent	78	62	69
Urine	Neg.	Neg.	Neg.
Cultures			
Blood	Neg.	Neg.	Neg.
Sputum (tubercle bacilli)	Neg.	Neg.	Neg.
Guinea pig inoc.	Neg.	Neg.	Neg.
Skin tests			
Histoplasmin	Neg. (Oct. 17)	Neg. (Oct. 16)	Not done
Blastomycin	Neg. (Oct. 24)	Not done	Not done
Coccidioidin	Neg. (Oct. 24)	Not done	Not done
Tuberculin	Neg.	Not done	Not done
Agglutination tests performed with Typhoid A and B, Brucella, Tularemia, and Proteus X19 antigens were negative in all three individuals			

E. E., who appeared more acutely ill, was admitted to Mercy Hospital October 9 where he remained until October 25. A. E., Jr., was admitted October 10 for six days. Mr. A. E., Sr., continued to have symptoms but remained at home until October 25, when he developed thrombophlebitis of the left leg. The clinical, physical, and pertinent laboratory findings of these individuals while at Mercy Hospital are shown in Table I. Although the symptoms in all three individuals were pronounced, there were few physical findings. Their white blood

cell counts were elevated and sedimentation rates increased. In spite of the marked pulmonary infiltrates, as shown by chest x-ray, the only abnormality noted on physical examination was râles. Skin tests with histoplasmin carried out on E. E. (October 17) and A. E., Jr. (October 16) gave negative reactions. Coccidioidin and tuberculin skin tests were also negative on E. E. on October 24 (Table I). While in the hospital, E. E. was given penicillin and streptomycin, but he had already begun to improve symptomatically before admission. At the time of discharge October 25 his only symptom was a slight elevation of the afternoon temperature. His cough had disappeared, his appetite had returned, and he was gaining weight. A. E., Jr., at the time of admission, was definitely improved and at the time of discharge, October 16, was afebrile. On the basis of the clinical and laboratory findings at Mercy Hospital, it was thought probable that the father and his two sons had a fungus infection of the lungs.

E. E. was readmitted to Hines Veterans Hospital, Chicago, for five days (October 27 to November 2) for further study. He was afebrile. Again, x-ray of the chest revealed widespread pulmonary infiltrates. At this admission the white blood cell count was 14,000 with 57 per cent polymorphonuclear leukocytes. Skin tests with coccidioidin and blastomycin were again negative, but the histoplasmin skin test which was previously negative was now mildly positive. The clinical and laboratory findings at the Veterans Hospital confirmed the previous impression that E. E. had a pulmonary fungus infection, the nature of which was not determined.

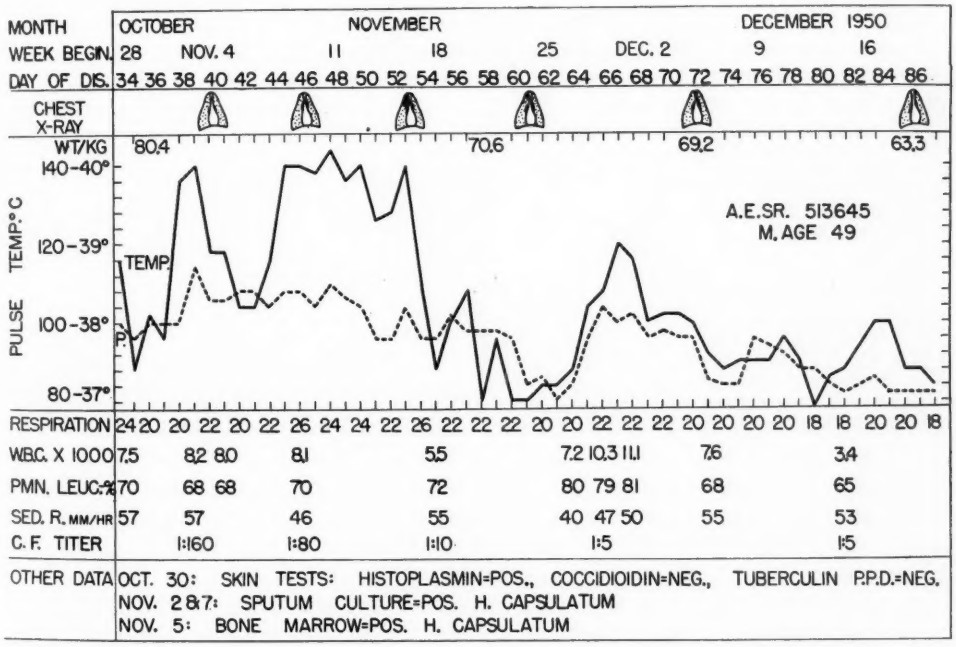
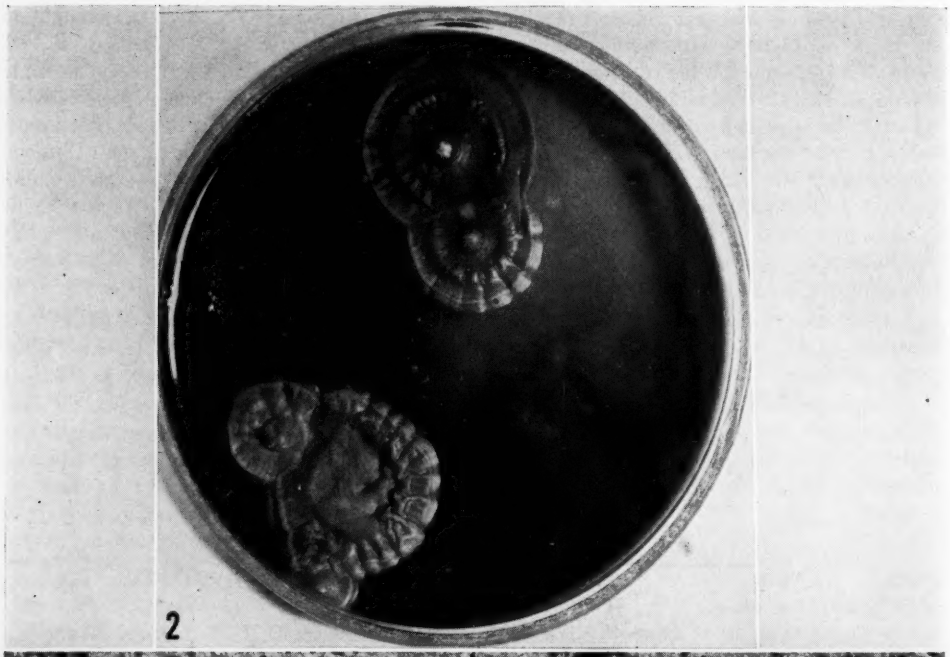


Fig. 1.—Temperature and pulse curves of A. E., Sr., and certain laboratory data obtained during his stay at the University of Chicago Clinics Hospital.

Because Mr. A. E., Sr., appeared gravely ill with a febrile illness of undetermined etiology associated with pulmonary infiltrates, splenomegaly, hepatomegaly, and thrombophlebitis, he was transferred on Oct. 28, 1950, to the University of Chicago Clinics for further study and care. The temperature and pulse curves and other pertinent laboratory data obtained during this admission are shown in Fig. 1. On admission he was febrile and appeared acutely ill. He stated that he had lost 20 pounds since the onset of his illness five weeks previously. He had no respiratory symptoms except for a morning cough, but complained



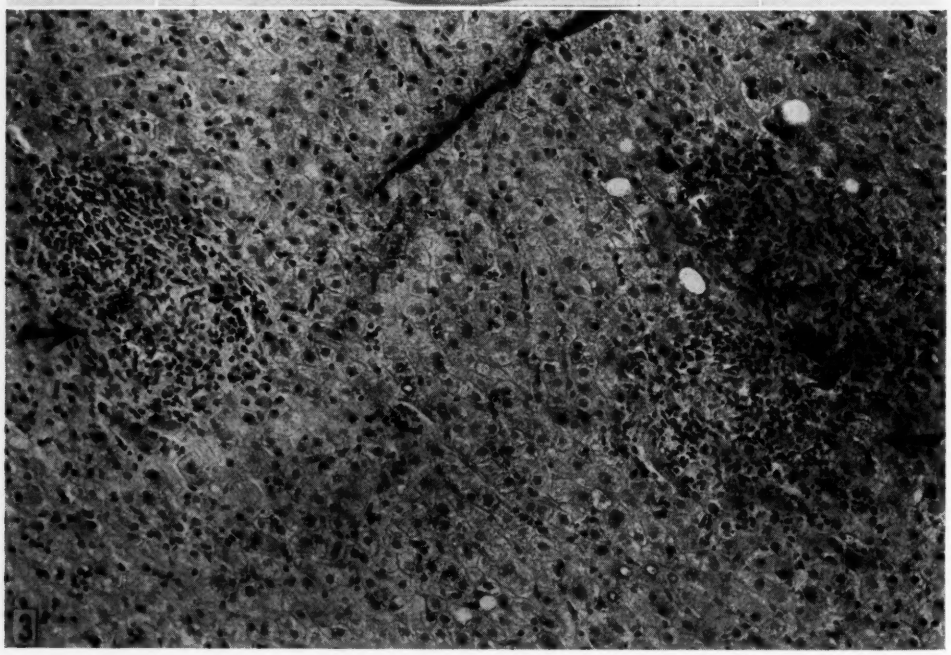


Fig. 2.—Bone marrow culture of A. E., Sr., on ninth hospital day, showing characteristic waxy cerebriform growth of *H. capsulatum* on blood-enriched agar at room temperature. (Slightly reduced.)

Fig. 3.—Granulomatous lesions in the biopsy specimen of the liver of A. E., Sr. No histoplasma organisms were seen. (Hematoxylin and eosin stain. ×100; reduced ⅓.)

of nausea, vomiting, and loose stools. On examination scattered respiratory wheezes and coarse rhonchi were heard throughout both lungs. The pulse was 100, but the heart sounds were good. The blood pressure was 100 systolic and 55 diastolic. There was no lymphadenopathy and the liver and spleen could not be felt although they were thought to be enlarged while at Mercy Hospital. The left leg was warm and swollen with 2 plus pitting edema. There was left calf tenderness. The white blood cell count was 7,500 with 75 per cent neutrophils. The sedimentation rate was 57 mm. per hour (Wintrobe). The hemoglobin was 11.5 Gm. The urine showed a trace of albumin. An x-ray of the chest on October 30 (Fig. 4) again showed extensive noncalcified infiltrates throughout both lung fields and no apparent change from the one taken October 10 at Mercy Hospital. A skin test with histoplasmin* (1:100 dilution) was strongly positive but negative with coccidioidin* (1:100 dilution) and tuberculin* (P.P.D. 0.0001 mg.). The complement fixation (C.F.) test employing the histoplasma yeast phase antigen† was positive in a serum dilution of 1:160. Agglutination tests employing the typhoid dysentery group antigens were negative. The cold agglutination test was also negative. Agglutination antibody titer with the brucella antigen was negative.

Routine blood, stool, and sputum cultures yielded no pathogenic organisms. Cultures and guinea pig inoculation of sputum and gastric washings for tubercle bacilli were negative. Repeated cultures of the blood, stools, sputum, and bone marrow for fungi were negative except for sputum samples collected on the thirty-ninth and forty-fourth days of illness, and bone marrow obtained on the forty-second day which yielded *H. capsulatum* organisms (Fig. 2). Direct examination of the bone marrow revealed no abnormality. Direct examination of the sputum failed to reveal the fungus. A liver punch biopsy taken on the forty-fifth day of illness showed an infectious granuloma, but no *H. capsulatum* organisms were observed on direct examination or culture (Fig. 3). On the fifty-first day of illness the cephalin flocculation test showed a 3 plus reaction, and the thymol turbidity value was 9.0 units. Plasma protein determinations revealed an elevated globulin (3.85 Gm. per cent). On the eighty-second day, one week before discharge, the globulin was still elevated (3.59 Gm. per cent) but the cephalin and thymol turbidity tests were within normal limits (1 plus and 3.4 units, respectively).

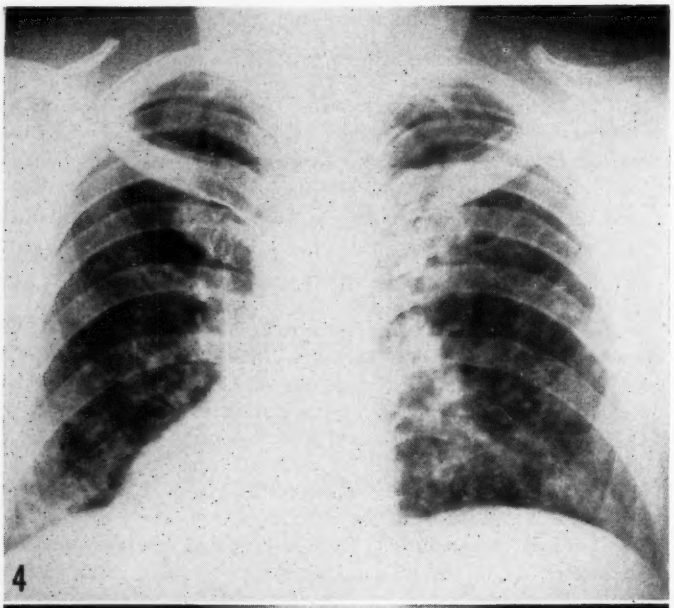
Mr. E.'s illness was a stormy one (Fig. 1). The thrombophlebitis of the left leg gradually subsided with application of dry heat and leg elevation. At the end of the first week in the hospital, however, he developed right upper quadrant and right lower chest pain which was worse on breathing. This correlated with a temperature rise and increasing râles in the right lung. Penicillin (800,000 units a day for six days) and Aureomycin (4 Gm. a day for another two days) had no effect on the temperature or symptoms. Following the liver biopsy the pain in the right chest intensified and radiated to the right shoulder. Physical signs of pneumonic consolidation developed in the right upper chest and the sputum became grossly bloody. Still no pathogenic organisms were isolated on culture. An x-ray of the chest at this time (Fig. 1) showed consolidation in the right upper lung field with scattered infiltrates throughout both lung fields. After ten days of high spiking fever, chills, and right chest pain these symptoms gradually subsided.

On the sixty-sixth day of illness (Fig. 1) Mr. E. developed pain and swelling in the calf of his right leg, and again a diagnosis of thrombophlebitis was made. For the next five days the temperature remained elevated. Heparin and Dicumarol were given and the prothrombin time was maintained at 50 per cent of normal. Dry heat was also applied.

Throughout most of his hospital stay Mr. E. had nausea, marked anorexia, and continued weight loss although he was given a high-vitamin, high-calorie diet with frequent glucose administrations by vein. However, with the subsidence of the right thrombophlebitis, his symptoms of anorexia disappeared and general improvement became pronounced. Mr. E. was discharged on Dec. 21, 1950, after 55 days of hospitalization and 86 days of illness.

During this period he had lost approximately 40 pounds in weight. Repeated x-rays of the chest revealed essentially no change in the size and character of the disseminated pulmo-

*The skin test antigens in the above dilutions were employed throughout this study.
†This type antigen was the only one employed in the C.F. test.



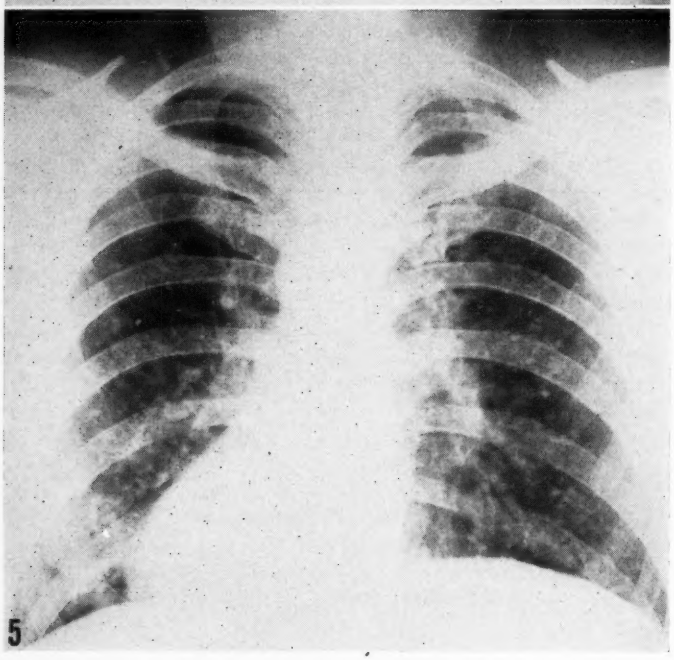


Fig. 4.—Chest x-ray of A. E., Sr., taken Oct. 30, 1950, six weeks after onset of illness, shows ill-defined, noncalcified, pulmonary infiltrates scattered throughout both lung fields.

Fig. 5.—Chest x-ray of A. E., Sr., taken Oct. 3, 1953, approximately three years after the onset of illness, shows some resolution of the infiltrates, but many fibrotic nodules are seen throughout the lung fields and especially in the region of the left lower lobe. Only a few of the nodules have begun to calcify.

nary infiltrates from the time of onset of illness to the time of discharge from the hospital. During the period of illness, the white blood cell counts showed only slight increase in numbers, while the sedimentation rate remained elevated (Fig. 1). He maintained a mild anemia throughout his hospital stay. At the time of discharge the red blood cell count was 3.79 million and the cell volume was 35 per cent. The C.F. antibody titer had fallen from 1:160 on the thirty-sixth day of illness to 1:5 on the eighty-second day (Fig. 1).

Although Mr. E.'s two sons, E. E. and A. E., Jr., had been clinically well for two to three weeks, they were admitted to the University of Chicago Clinics from Nov. 9 to 11, 1950, for further laboratory study. The physical examinations revealed no abnormal findings. X-rays of the chest again showed extensive ill-defined noncalcified pulmonary infiltrates (Figs. 6 and 8). Cultures of the blood, bone marrow, and gastric washings for fungi and tubercle bacilli were negative. On skin testing with tuberculin there was no reaction, but the reaction with histoplasmin was markedly positive. The C.F. tests were positive in serum dilutions of 1:120 (E. E.) and 1:160 (A. E., Jr.).

Nonclinical Illnesses.—During the period of illness of the father and his two sons the other members of the family had remained well. However, during the month of November, 1950, Al. E., D. E., J. E., M. E., and S. E. were examined for evidence of current or previous infections due to the histoplasma fungus. The physical examinations revealed no abnormal findings except that the heart of the mother (S. E.) was on the right and Al. E. showed some achondroplastic skeletal changes which had been present since birth. All had normal blood cell counts, but the sedimentation rate in the case of M. E. was elevated. The urine examinations were negative.

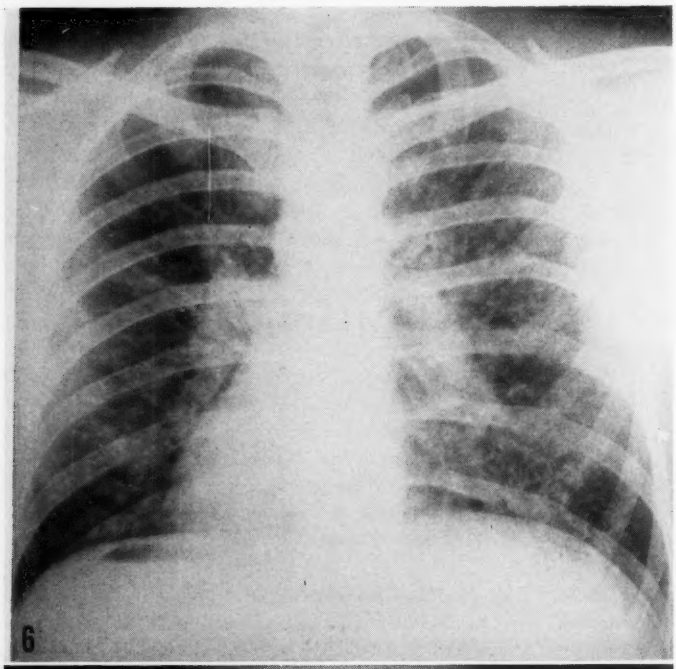
X-ray of the chest of M. E. revealed a left hilar infiltrate (Fig. 10), that of Al. E. showed an increased hilar shadow on the right and a small infiltrate in the right lower lobe (Fig. 12). The x-ray of J. E. showed two infiltrates in the right, middle, and lower lung fields (Fig. 14). Those of the daughter-in-law, D. E., and the mother, S. E., showed no infiltrates, but the x-ray of the latter revealed a dextrocardia as was noted on physical examination. The daughters (Al. E. and M. E.) and the son (J. E.) showed positive reactions with the histoplasmin antigen but negative reactions to tuberculin. The mother (S. E.) and daughter-in-law (D. E.) reacted negatively to both antigens.

The serum of M. E. showed C.F. antibodies in a dilution of 1:20 while that of Al. E. showed a titer of 1:10. The C.F. tests on the sera of J. E., S. E., and D. E. were negative.

A summary of the pertinent laboratory findings on the E. family at the first survey are shown in Table II. It can be seen that of the eight individuals only three had febrile illnesses. The father's was most severe, lasting for approximately three months. Six of the eight showed noncalcified pulmonary infiltrates by x-ray. Sputum and bone marrow cultures from the father yielded *H. capsulatum.* Skin tests with histoplasmin showed that all reacted positively except in the case of the mother and daughter-in-law. None reacted to tuberculin. Sera from five of the eight individuals showed complement fixing antibodies with the yeast-phase histoplasma antigen. The highest titers were found in the sera of the father and his two sons who had febrile illnesses.

THE FOLLOW-UP

After Mr. E. was discharged from the hospital, the members of the family were seen as a group in the clinics at two to six months' intervals. At each visit histories were taken and physical examinations were done. Laboratory procedures included blood and urine examinations, x-ray of the chest, skin testing with histoplasmin, tuberculin, and coccidioidin, and serologic C.F. test with the yeast-phase antigen.



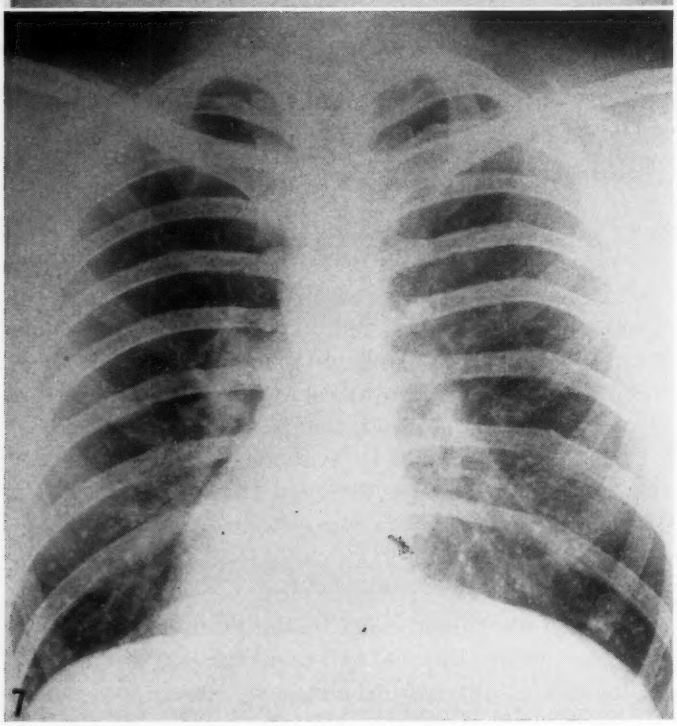


Fig. 6.—Chest x-ray of E. E., taken Nov. 9, 1950, eight weeks after onset of illness, shows numerous ill-defined, noncalcified densities throughout both lung fields.

Fig. 7.—Chest x-ray of E. E., taken Oct. 3, 1953, approximately three years after onset of illness, shows extensive miliary calcifications throughout both lung fields.

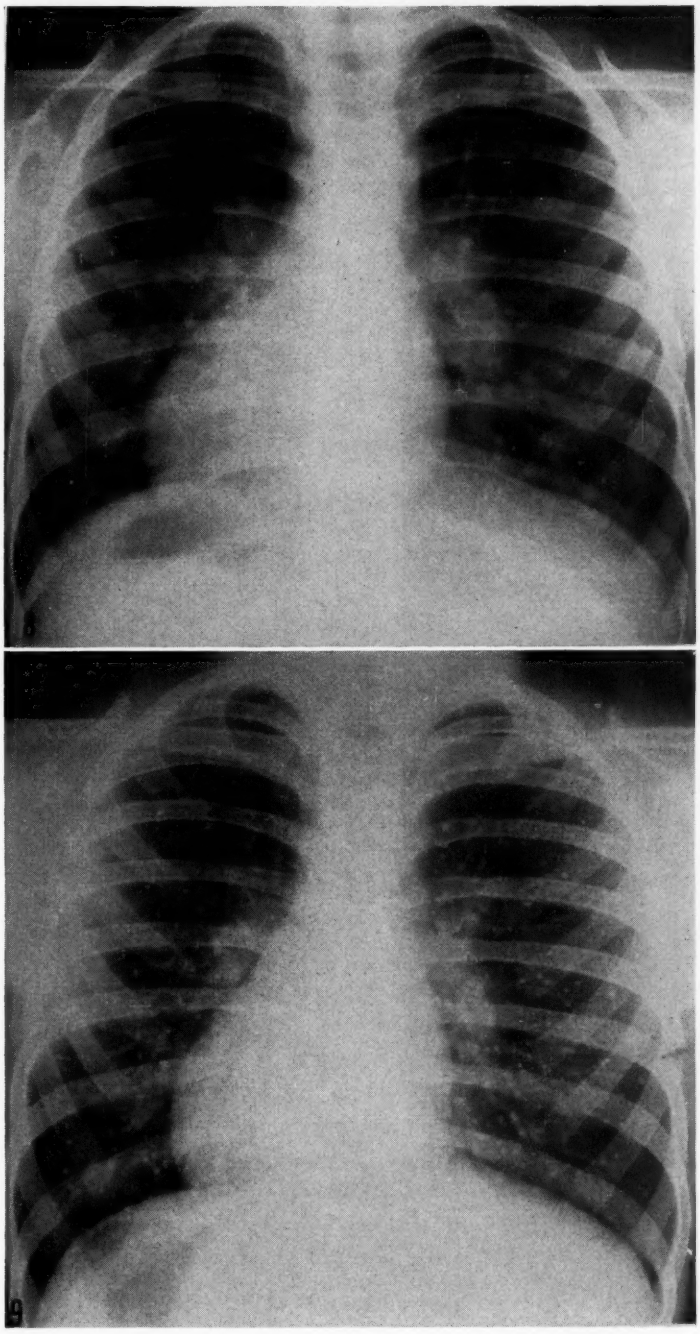


Fig. 8.—Chest x-ray of A. E., Jr., taken Nov. 10, 1950, approximately eight weeks after onset of illness, shows ill-defined, noncalcified densities throughout both lung fields.

Fig. 9.—Chest x-ray of A. E., Jr., taken Oct 3, 1953, approximately three years after the onset of illness, shows extensive miliary calcification throughout both lung fields.

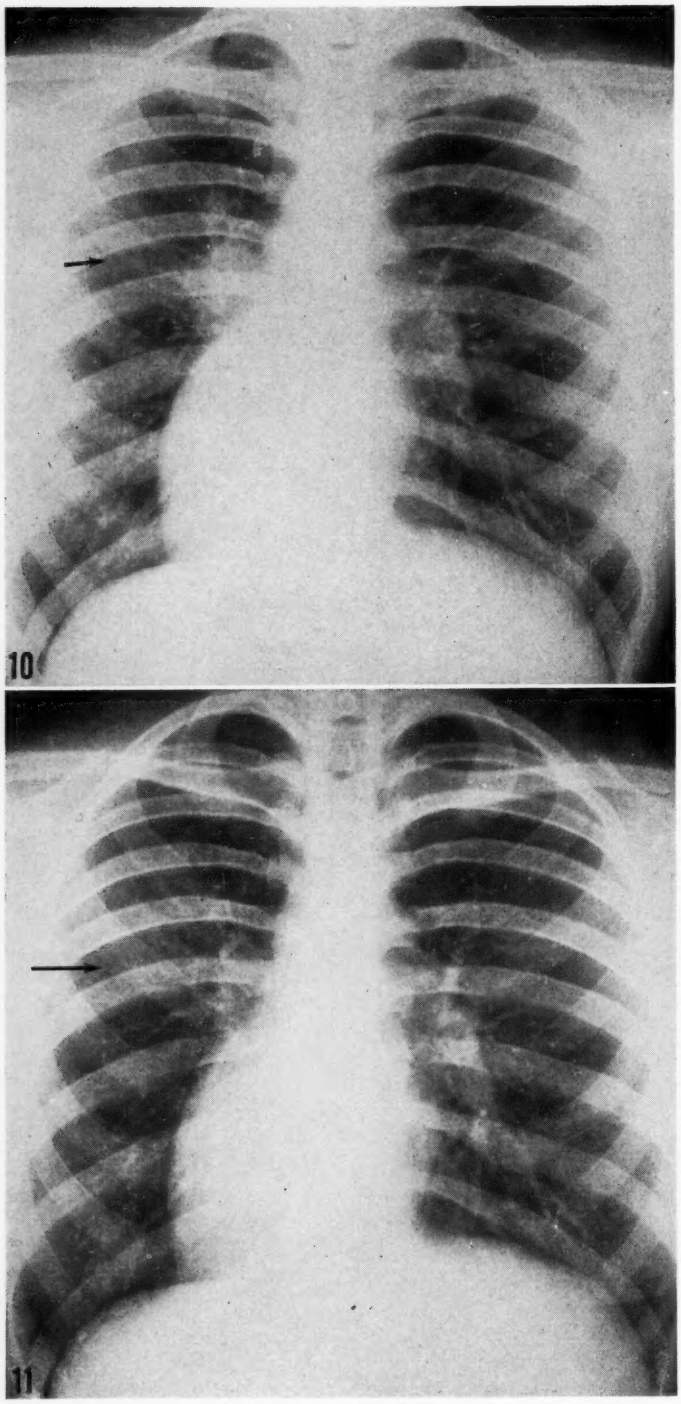


Fig. 10.—Chest x-ray of M. E., taken Nov. 11, 1950, shows a noncalcified left hilar infiltrate.

Fig. 11.—Chest x-ray of M. E., taken April 23, 1951, shows resolution of the left hilar infiltrate.

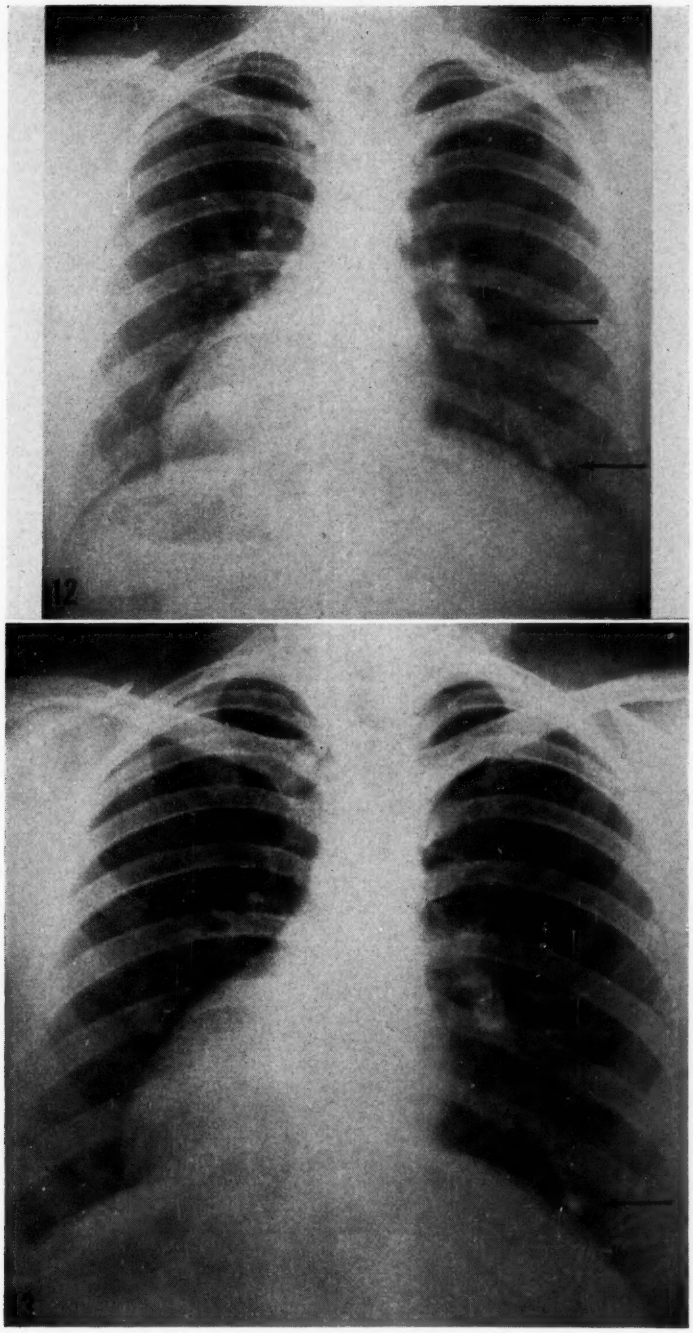


Fig. 12.—Chest x-ray of Al. E., taken Nov. 11, 1950, shows right hilar and lower lobe infiltrates.

Fig. 13.—Chest x-ray of Al. E., taken Oct. 6, 1953, shows multiple calcifications in right hilar area and in the nodule in the right lower lobe.

TABLE II. INITIAL LABORATORY FINDINGS ON MEMBERS OF THE E. FAMILY TAKEN DURING PERIOD FROM OCT. 30 TO DEC. 1, 1950

INITIAL SEX & AGE (YEARS)	FEBRILE ILLNESS DURATION	X-RAY OF CHEST	CULTURES FOR H. CAPSULATUM			SPUTUM CULTURES FOR TUBERCLE BACILLI	SKIN TESTS		C. F. ANTIBODY TITER
			SPUTUM	BONE MARROW	BLOOD		HISTO-PLASMIN	TUBER-CULIN	
A. E., Sr. M—49	86 days	Extensive ill-defined noncal. infiltrates	Pos.	Pos.	Neg.	Neg.	Pos.	Neg.	1:160
E. E. M—21	28 days	Extensive ill-defined noncal. infiltrates	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	1:320
A. E., Jr. M—5	14 days	Extensive ill-defined noncal. infiltrates	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	1:160
M. E. Fe—17	None	Left upper hilar infiltrate		Not done		Not done	Pos.	Neg.	1:20
Al. E. Fe—19	None	Single infiltrate right lower lobe		Not done		Not done	Pos.	Neg.	1:10
J. E. M—24	None	Two infiltrates right lung		Not done		Not done	Pos.	Neg.	Neg.
S. E. Fe—42	None	No infiltrate or calcification		Not done		Not done	Neg.	Neg.	Neg.
D. E. Fe—24	None	No infiltrate or calcification		Not done		Not done	Neg.	Neg.	Neg.

A. E., Sr.—Mr. E. had lost approximately 40 pounds in weight during the course of his illness. However, at the time of his first return visit (Table III) he had begun to gain weight and continued to do so for the next six months. By April, 1951, Mr. E. had regained strength sufficiently to carry on the routine farm activities, although he complained of

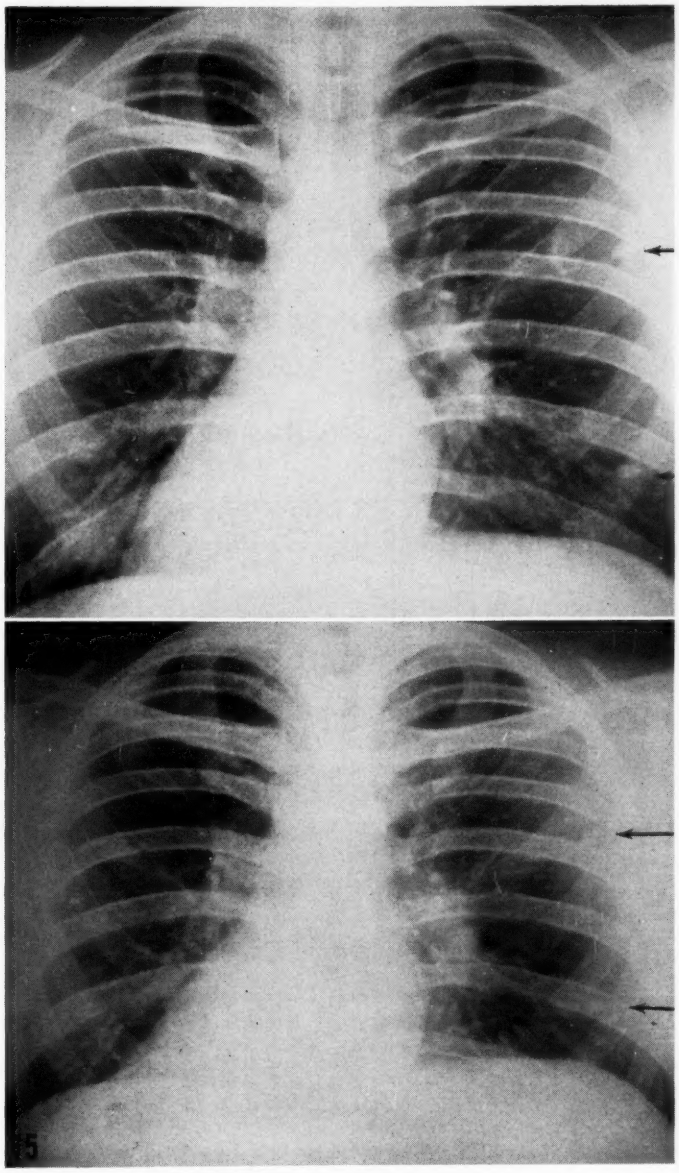


Fig. 14.—Chest x-ray of J. E., taken Jan. 21, 1951, shows two noncalcified infiltrates in the right, middle, and lower lung fields.

Fig. 15.—Chest x-ray of J. E., taken Oct. 25, 1952, shows no change in the character of the infiltrates in the right lung field after twenty-one months.

intolerance to heat and excessive sweating on physical exertion. During the follow-up period of observation he has had no major intercurrent illnesses. The hemoglobin and red cell values have remained approximately the same. The white blood cell counts have remained slightly elevated with the percentage of polymorphonuclear leukocytes being within the normal

TABLE III. LABORATORY FINDINGS OF A. E., SR., DURING FOLLOW-UP PERIOD, JAN. 12, 1951, TO OCT. 3, 1953

DATE	WEIGHT (KG.)	CHEST X-RAY	HEMOGLOBIN (GM. %)	BLOOD EXAMINATIONS				C. F. ANTIBODY TITER	SKIN TESTS		
				R. B. C. MIL./CU. ML.	W. B. C. CU. ML.	PMN LEUK./%	SED. RATE MM./HR.		HISTO-PLASMIN	COCCID-IOIDIN	TUBER-CULIN
1/12/51	64.0	Noncalcified infiltrates	13.0	4.31	7,500	47	47	Neg.	Pos.	Neg.	Neg.
2/23/51	69.0	Noncalcified infiltrates	13.3	-	10,500	51	45	Neg.	Pos.	Neg.	Neg.
4/23/51	75.8	Noncalcified infiltrates	13.5	4.29	10,100	52	33	Neg.	Pos.	Neg.	Neg.
6/23/51	74.6	Clearing	14.6	4.55	8,500	51	20	Neg.	Pos.	Neg.	Neg.
8/30/51	75.8	Infiltrates more fibrotic	13.4	4.07	6,900	55	26	Neg.	Pos.	Neg.	Neg.
1/26/52	79.0	Infiltrates more fibrotic	12.9	4.13	10,100	67	12	Neg.	Pos.	Neg.	Pos.
4/19/52	78.6	Infiltrates more fibrotic	14.0	4.19	6,500	51	30	Neg.	Pos.	Neg.	Pos.
10/25/52	78.2	Calcification of infiltrates	13.8	4.42	8,300	56	35	Neg.	Pos.	Neg.	Pos.
4/11/53	75.6	Calcification of infiltrates	14.4	4.54	8,400	46	22	Neg.	Pos.	Neg.	Pos.
10/3/53	74.6	More calcification of infiltrates	14.1	4.60	10,800	67	35	Neg.	Pos.	Neg.	Pos.

TABLE IV. LABORATORY FINDINGS OF E. E. DURING INITIAL VISIT AND FOLLOW-UP PERIOD, NOV. 9, 1950, TO OCT. 3, 1953.

DATE	WEIGHT (KG.)	CHEST X-RAY	HEMOGLOBIN (GM. %)	BLOOD EXAMINATIONS				C. F. ANTIBODY TITER	SKIN TESTS		
				R. B. C. MIL./CU. ML.	W. B. C. CU. ML.	PMN LEUK./%	SED. RATE MM./HR.		HISTO-PLASMIN	COCCID-IOIDIN	TUBER-CULIN
11/ 9/50	58.7	Noncalcified infiltrates	12.0	4.15	7,700	67	29	1:320	Pos.	Neg.	Neg.
12/ 1/50	61.4	Noncalcified infiltrates	15.5	4.95	13,400	74	31	1:160	Pos.	Neg.	Neg.
1/12/51	61.8	Noncalcified infiltrates	14.0	4.52	11,000	77	16	1:80	Pos.	Neg.	Neg.
2/23/51	61.0	Fibrotic infiltrates	14.8	-	15,100	75	22	1:40	Pos.	Neg.	Neg.
4/23/51	64.5	Fibrotic infiltrates	14.4	4.30	10,700	71	25	Neg.	Pos.	Neg.	Neg.
6/23/51	62.3	Infiltrates more fibrotic	16.2	5.08	12,500	76	6	Neg.	Pos.	Neg.	Neg.
8/30/51	61.8	Fibrotic infiltrates	13.9	4.71	10,300	75	15	Neg.	Pos.	Neg.	Neg.
1/26/52	63.2	Calcification of infiltrates	14.8	4.58	10,800	68	18	Neg.	Pos.	Neg.	Neg.
4/19/52	67.4	More calcification infiltrates	14.7	4.38	12,000	80	9	Neg.	Pos.	Neg.	Neg.
10/25/52	63.6	More calcification infiltrates	14.3	4.35	13,000	75	19	Neg.	Pos.	Neg.	Neg.
4/11/53	67.8	More calcification infiltrates	14.5	4.45	9,200	78	15	Neg.	Pos.	Neg.	Neg.
10/ 3/53	64.1	More calcification infiltrates	14.3	4.36	9,200	80	11	Neg.	Pos.	Neg.	Neg.

TABLE V. LABORATORY FINDINGS OF A. E., JR., AT THE INITIAL VISIT AND DURING FOLLOW-UP PERIOD, NOV. 9, 1950, TO OCT. 3, 1953

DATE	WEIGHT (KG.)	CHEST X-RAY	HEMOGLOBIN (GM. %)	BLOOD EXAMINATIONS				C. F. ANTIBODY TITER	SKIN TESTS		
				R. B. C. MIL./CU. ML.	W. B. C. CU. ML.	PMN LEUK./%	SED. RATE MM./HR.		HISTO-PLASMIN	COCCID-IOIDIN	TUBER-CULIN
11/ 9/50	17.7	Noncalcified infiltrates	12.6	4.48	8,100	56	38	1:160	Pos.	Neg.	Neg.
12/ 1/50	18.6	Noncalcified infiltrates	12.8	4.37	9,000	50	37	1:80	Pos.	Neg.	Neg.
1/12/51	19.1	Noncalcified infiltrates	11.7	4.11	8,700	63	34	1:40	Pos.	Neg.	Neg.
2/23/51	19.1	Fibrosis of infiltrates	11.3	4.10	7,800	72	30	1:20	Pos.	Neg.	Neg.
4/23/51	19.6	Fibrosis of infiltrates	11.4	3.89	9,200	64	47	1:20	Pos.	Neg.	Neg.
6/23/51	19.6	Some calcification	12.5	3.87	5,300	-	12	Neg.	Pos.	Neg.	Neg.
8/30/51	19.2	More calcification	11.5	3.86	9,700	69	31	Neg.	Pos.	Neg.	Neg.
1/26/52	20.8	Calcification complete	14.6	4.47	7,400	50	34	Neg.	Pos.	Neg.	Neg.
4/19/52	20.8	Calcification complete	11.8	3.82	7,900	51	19	Neg.	Pos.	Neg.	Neg.
10/25/52	21.4	Calcification complete	12.5	4.29	7,500	56	15	Neg.	Pos.	Neg.	Neg.
4/11/53	23.2	Calcification complete	12.6	4.18	9,300	65	16	Neg.	Pos.	Neg.	Neg.
10/ 3/53	23.7	Calcification complete	13.1	4.39	7,600	52	15	Neg.	Pos.	Neg.	Neg.

range. The sedimentation rate gradually became lower until January, 1952, when it reached a normal value. Thereafter, the readings showed moderately elevated values (Table III) compared to the period of febrile illness (Fig. 1). Liver function tests, including plasma protein determinations, showed normal values as of April, 1953.

The C.F. test at the time of discharge showed a value of 1:5 (Fig. 1), but on the first return visit three weeks later (Jan. 12, 1951) it was negative and has remained so. The histoplasmin skin test has, on the other hand, been consistently positive. The repeated coccidioidin skin tests have been negative. The tuberculin skin test converted from a negative to a positive reaction, January, 1952.* (Table III.)

In spite of the marked involvement of the lungs as shown by x-ray, the physical signs have been normal throughout the period of observation. There was essentially no change in the x-ray appearance of the lungs during the first six months after leaving the hospital. Since this time, however, there has been resolution of some of the infiltrates, but many remaining have become more discrete and fibrotic. Since October, 1952, approximately twenty-four months after onset of his illness, a few of the fibrotic nodules have undergone calcification, but as shown in the last x-ray, taken approximately three years after onset of illness, the majority remain uncalcified (Table III, Fig. 5).

E. E.—As previously stated, E. E. was quite well when examined Nov. 9, 1950, although he had lost several pounds in weight during his recent illness. During the following period, he has gradually regained his weight and strength and has participated in unlimited activity on the farm or as an employee in a steel mill in Gary, Ind. He has had no major intercurrent illnesses. The laboratory findings, beginning with the initial survey, obtained over the past three years, are shown in Table IV. At the first examination the hemoglobin and red cell values were slightly below the normal range. The white blood cell counts with the exception of the first one have been consistently elevated. The sedimentation rate, although never very high, had returned to normal by June, 1951, and has shown values from 9 to 19 mm. per hour since then (Table IV). With recovery, the C.F. antibody titer which showed a value of 1:320 on November 9, approximately six weeks after the onset of his illness, fell and was negative by April, 1951. The skin test with histoplasmin has remained positive while the others have remained negative (Table IV).

As was the case with Mr. A. E., Sr., there were no abnormal findings on physical examination of the chest in spite of the marked involvement as seen by x-ray. The x-ray appearance of the lungs began to show improvement approximately four months (January, 1951) after onset of illness as indicated by a decrease in the number of irregularly defined, patchy infiltrates throughout the lungs. During the following year the densities became smaller and more discrete, and by January, 1952, approximately seventeen months after the onset of his illness, some had begun to calcify. By October, 1952, the majority of nodules showed some calcium deposition. Since this time there has been little change in the x-ray appearance of the chest. The most recent x-ray, taken approximately three years after onset of illness, is shown in Fig. 7.

A. E., Jr.—At the time of examination, Nov. 9, 1950, A. E., Jr., was clinically well and was not restricted in activity. His appetite had returned and he has steadily gained weight since this time (Table V). He has had several minor respiratory illnesses, chicken pox, and several bouts of streptococcal tonsillitis for which a tonsillectomy was performed June, 1953. Throughout the period of observation the white blood cell counts have remained slightly elevated but the sedimentation rate gradually returned to normal values.

C.F. antibodies which were present in a serum dilution of 1:160 in November, 1950, were no longer present when he was examined in June, 1951, some nine months after onset of illness (Table V). The histoplasmin skin test, however, has been consistently positive while the other skin tests have been negative (Table V).

As was the case of A. E., Sr., and E. E., the findings in the lungs on physical examination have shown no abnormalities in spite of the marked involvement shown by x-ray. Little or no change was noted in the x-ray appearance of the disseminated noncalcified densities in the lungs during the first four months after onset of illness. By February, 1951, the densities

*Inquiry revealed that the most probable "contact" case of active tuberculosis was a salesman from whom the E. family purchased a television set during the late summer of 1951.

had become smaller, more discrete and fibrotic, and by June, 1951, nine months after onset of illness, many showed calcified centers (Table V). Thereafter, rapid calcification of the remaining nodules occurred so that by January, 1952, sixteen months after onset of illness, healing of the lung lesions appeared to be complete. During the next twenty months essentially no change has been noted in the x-ray appearance of the lungs. The most recent one is shown in Fig. 9.

M. E.—Throughout the period of observation M. E. has remained well. Since the first examination she has married (April, 1951) and has borne a healthy child (June, 1952). The C.F. test done at the first survey was positive in a serum dilution of 1:20, but was negative when repeated five months later. The skin test for histoplasmosis has remained positive, and the tuberculin and coccidioidin skin tests have been consistently negative. The left upper hilar infiltrate noted Nov. 11, 1950 (Fig. 10), underwent resolution during the next five months so that it was not seen in the x-ray taken in April, 1951 (Fig. 11). X-rays taken at six-month intervals since this time have shown no abnormalities or calcification in the area of involvement.

Al. E.—At the first survey, Nov. 11, 1950, this 19-year-old daughter was also clinically well. At this time the C.F. antibody titer was 1:10. Five months later it was negative. The histoplasmin skin test has been consistently positive while tuberculin and coccidioidin skin tests with antigens have been consistently negative. She has continued to remain well. The initial x-ray of the chest showed a right hilar infiltrate and an irregularly shaped density with a calcified center in the right lower lung field (Fig. 12). During the three-year period of observation the x-ray appearance has remained essentially unchanged. In the most recent x-ray of the chest, Oct. 6, 1953, it would appear that more calcification has developed in the hilar area and in the lesion in the right lower lung field.

J. E.—J. E. was well at the first examination and has remained so during the period of observation. Repeated C.F. tests have been negative, but the skin test with the histoplasmin antigen has been consistently positive while those employing tuberculin and coccidioidin antigens have always been negative. The initial x-ray examination of the chest revealed two-well-circumscribed noncalcified densities in the right, middle, and lower lung field. These have shown no change during the period of observation. The most recent x-ray of the chest, taken Oct. 6, 1953, shows no change in the nodules when compared to those seen in the x-rays taken in January, 1951 (Fig. 14), and October, 1952 (Fig. 15).

S. E. and D. E.—These individuals have been well during the period of observation. The physical examinations, blood counts, C.F. tests, x-ray examinations of the chest, and skin tests with histoplasmin and coccidioidin antigens on these individuals have been consistently negative during the three-year period of observation. Likewise, D. E. reacted negatively with tuberculin, but Mrs. S. E., since January, 1952,* has reacted in a positive manner to this antigen.

In Table VI is given a summary of the most recent findings (October, 1953) on the E. family as shown by physical examination, chest x-ray, C.F., and skin tests. As has been the case for the past two and a half years, all are clinically and physically well. C.F. antibody tests are all negative. The six members initially reacting in a positive manner to the histoplasmin continue to do so after three years. The two reacting in a negative manner also continue to do so. The parents (A. E., Sr., and S. E.) since January, 1952, have shown positive tuberculin reactions as well. None has reacted to the coccidioidin skin test.

X-rays of the chest of the father show only gradual improvement in the appearance of the pulmonary densities with only beginning calcification (compare Figs. 4 and 5); while x-rays of the chest of E. E. and A. E., Jr., show healing with extensive calcification (compare Figs. 6 and 7 and 8 and 9). The fibrotic nodules in the lungs of J. E. did not change in appearance during the

*See previous footnote in regard to probable contact with tuberculosis.

period of observation (compare Figs. 14 and 15) while the right hilar density and nodule in the right lower lobe of Al. E. showed increased calcification (compare Figs. 12 and 13). The left hilar infiltrate in the lungs of M. E. resolved after five months and at the most recent survey shows no evidence of residual calcification (compare Figs. 10 and 11). The lungs of S. E. and D. E. have remained clear.

TABLE VI. STATUS (OCTOBER, 1953) OF E. FAMILY THREE YEARS AFTER ONSET OF ILLNESS AS SHOWN BY CHEST X-RAY, COMPLEMENT FIXATION AND SKIN TESTS

INITIAL	CLINICAL STATUS	X-RAY OF CHEST	SKIN TESTS			C. F. ANTIBODY TITER
			HISTOPLASMIN	TUBERCULIN	COCCIDIOIDIN	
A. E., Sr.	No symptoms	Extensive infiltrates, slight calcification	Pos.	Pos.	Neg.	Neg.
E. E.	No symptoms	Clearing with extensive calcification of infiltrates	Pos.	Neg.	Neg.	Neg.
A. E., Jr.	No symptoms	Extensive miliary calcification	Pos.	Neg.	Neg.	Neg.
M. E.	No symptoms	Disappearance of hilar infiltrate	Pos.	Neg.	Neg.	Neg.
Al. E.	No symptoms	Calcification of hilar infiltrate and RLL infiltrate	Pos.	Neg.	Neg.	Neg.
J. E.	No symptoms	No change in infiltrates	Pos.	Neg.	Neg.	Neg.
S. E.	No symptoms	No infiltrates or calcification	Neg.	Pos.	Neg.	Neg.
D. E.	No symptoms	No infiltrates or calcification	Neg.	Neg.	Neg.	Neg.

EPIDEMIOLOGICAL STUDIES

When the diagnosis of pulmonary histoplasmosis was established in the E. family, a survey of possible sources of the fungus on the farm* was made. Among other activities Mr. A. E., Sr., and his two sons had cleaned an unused silo during the first week in September, 1950, approximately three to four weeks prior to the onset of their illnesses. The silo had not been used to store silage for eighteen years, but had, on the other hand, been employed as an ice house up until six years before it was purchased by the E. family. The material removed from the silo, shown in Fig. 18, consisted of dry sawdust, wind-blown soil, and old corncobs. A. E., Jr., then 5 years old, sat on the truck into which his father and brother, E. E., shoveled the material. Occasionally some would light on his head and he would retaliate by throwing handfuls in the air and back at them. During the process of cleaning, the air in the silo, as recalled by the father and E. E., became very "dusty."

The eldest son, J. E., and the two daughters, M. E., and Al. E., helped occasionally with the work. All had been in the silo and all were on the farm when the silo was cleaned. M. E. drove the tractor. The mother and daughter-in-law (D. E.) had not been in the silo but were on the farm when it was cleaned. The silo, thus, was considered the most likely site where the fungus might be found.

*The farm is located near Lowell, Ind., approximately sixty miles from Chicago. It was purchased and first occupied by the E. family during the month of November, 1948.

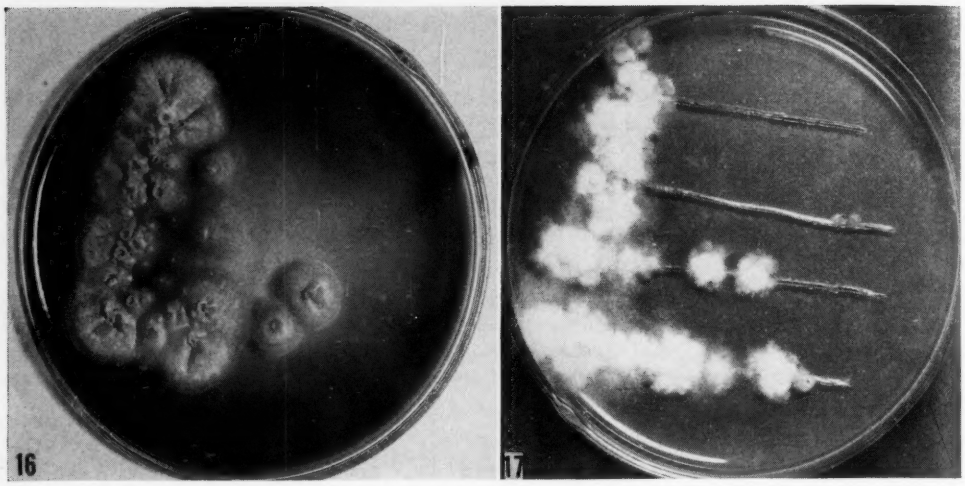


Fig. 16.—Photograph of *H. capsulatum* growing on blood agar, incubated at room temperature. The inoculum was spleen and liver mince of mice inoculated with soil suspension from the silo, Nov. 27, 1950.

Fig. 17.—Photograph of *H. capsulatum* growing on corn-meal agar incubated at room temperature. Inoculum was liver and spleen mince of mice inoculated with soil suspension from the silo, Nov. 27, 1950.

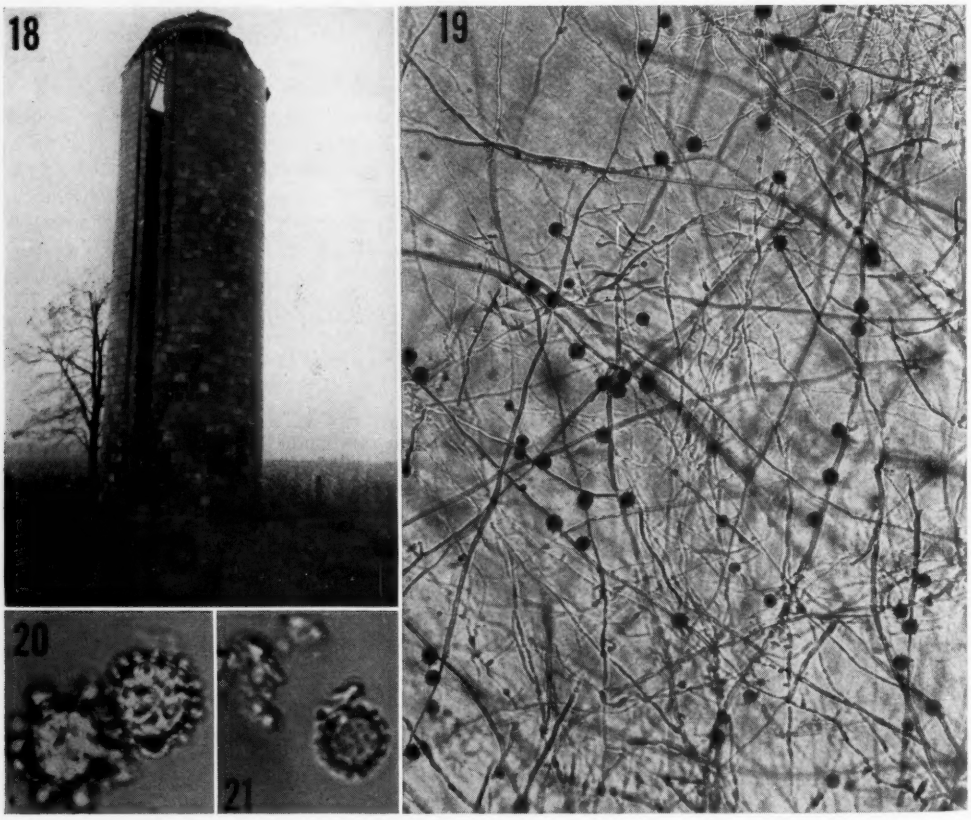


Fig. 18.—Photograph of unused silo which was found to be the source of the *H. capsulatum* fungus causing infections in the E. family.

Fig. 19.—Medium high-power photograph of mycelial growth of *H. capsulatum* showing chlamydospores. (×100.)

Fig. 20.—Photograph of unstained chlamydospore of *H. capsulatum* showing characteristic tuberculate border. (×600.)

Fig. 21.—Photograph of *H. capsulatum* tuberculate chlamydospore observed directly in soil suspension collected from the silo, June 25, 1951. (×600.)

In November, 1950, samples of soil were collected from different areas in the silo and other sites on the farm and subjected to examination for the histoplasma fungus, employing the methods suggested by Emmons.[4] Briefly, they consisted of inoculating the supernates of soil suspensions intraperitoneally into mice which had been placed on drinking water containing 0.5 mg. of Terramycin or Aureomycin per milliliter. One week after inoculation, the antibiotic drinking water was discontinued. After three to six weeks the animals were sacrificed and the spleens and livers of three to six mice were pooled, minced, and streaked on different fungus media.* The media included: 5 per cent sheep blood agar enriched with glucose and cysteine to which were added penicillin (20 units per milliliter) and streptomycin (40 units per milliliter), and Sabouraud's and corn meal agars. Material was inoculated on the three kinds of media and incubated at room temperature. Additional blood agar plates were incubated at 37° C. Cultures were not discarded as being negative until two months after inoculation. Suspected colonies of *H. capsulatum* growing on blood agar were transferred to corn meal agar and observed for characteristic mycelial growth with tuberculate chlamydospores.

During the November, 1950, survey, pooled samples of soil from the floor and wall-ledge† from the silo readily yielded the histoplasma fungus. Figs. 16 and 17 show the original soil isolate growing on blood agar and corn meal agar, respectively. A high magnification of the mycelial phase culture with chlamydospores is shown in Fig. 19. The characteristic unstained tuberculate chlamydospore is shown in Fig. 20. From time to time soil suspensions were examined directly for the presence of histoplasma spores and in several specimens these were seen. They were very prevalent in certain soil samples collected during the June, 1951, survey. Fig. 21 is a photograph of the characteristic tuberculate chlamydospore of *H. capsulatum* observed in a suspension of soil from the June, 1951, survey.

TABLE VII. NUMBER AND PERCENTAGE OF SILO SOIL SAMPLES POSITIVE FOR *H. capsulatum* FUNGUS DURING THREE YEARS OF STUDY

	WALL-LEDGE			FLOOR		
		POSITIVE			POSITIVE	
YEAR	NUMBER	NO.	PER CENT	NUMBER	NO.	PER CENT
1950-51	127	42	34.0	127	23	18
1952	112	8	7.1	112	12	10
1953	70	6	8.6	105	14	13

Soil from the silo has been surveyed for the presence of the fungus at four- to six-week intervals during the spring, summer, and fall months of the past three years, the last in October, 1953.‡ At each survey from 30 to 40 samples of soil from different areas in the silo were analyzed separately for the fungus. The percentage of positive cultures obtained from a given survey varied. Soil from some areas yielded the fungus consistently while soil from other areas has been repeatedly negative. The annual results of the three-year study are shown in Table VII. As can be seen, a great many more samples of soil obtained in 1950 and 1951 were positive for the histoplasma fungus than

*These media were also employed for the direct isolation of the fungus from the sputum and bone marrow of Mr. A. E., Sr.

†The ledge is formed by the cement foundation which is approximately six inches greater in thickness than the hollow tile wall and varies from six to ten inches above the dirt floor. It could be seen readily that during rainstorms soil from the floor was splashed onto the ledge.

‡When the *H. capsulatum* fungus was first isolated from the silo soil in November, 1950, it seemed important to continue to study the soil in the silo over several years. Mr. A. E., Sr., has kindly allowed us this privilege and made no further attempt to convert it to a granary.

were found in the subsequent years. The reasons for this are not readily apparent. Only one of forty samples of soil collected in October, 1953, yielded the fungus. Although the areas in the silo yielding positive soil samples have been examined carefully, mycelial growth of the fungus has not been observed.

DISCUSSION

The E. family epidemic of pulmonary histoplasmosis was the first to be reported in which the source of the fungus was readily identified in the immediate environment during the acute phase of illness.[1-3] However, Emmons[4] previously had isolated *H. capsulatum* from the soil in localities where animal infections were known, while Ajello and Zeidberg[5] were successful in recovering the fungus from the soil from areas showing a high prevalence of skin test sensitivity in the human population. More recently Gordon and associates[6, 7] have isolated *H. capsulatum* and other spores from soil and water by means of the membrane filter. Furcolow and Larsh[8] have recently reported the direct isolation of *H. capsulatum* from soil.

Furcolow and Grayston[9, 10] have made epidemiological and etiological observations on thirteen epidemics similar to that experienced by the E. family. Six of these outbreaks of pulmonary disease were previously reported by others as etiologically undetermined illnesses, and under a variety of names such as "acute miliary pneumonitis,"[11] "primary atypical pneumonia,"[12] "an unusual pulmonary disease,"[13] "pneumonitis of unknown etiology,"[14] "cave sickness: a new disease entity,"[15] and "an unusual type of pulmonary disease."[16] *H. capsulatum* was cultured from soil samples obtained in the immediate environment where the infections had occurred in eleven of the thirteen epidemics. Ninety-four of 116 persons involved in eleven of the epidemics were skin tested with histoplasmin. All reacted positively, whereas only a small portion reacted to coccidioidin, tuberculin, or blastomycin. Furcolow and Grayston[9] also demonstrated some serologic evidence of histoplasmosis in each epidemic. It is of significance that among 63 persons who exhibited some type of calcification, 44 had disseminated miliary calcification while the remaining 19 had single or multiple lesions.

In the E. family outbreak the two sons exposed to the infectious silo dust now show extensive miliary calcification of the lungs and the disseminated pulmonary lesions of the father are beginning to calcify after three years. Calcification took place most rapidly in the case of A. E., Jr., 5 years old. These observations support those of Furcolow and associates[17-20] and others[21, 22] that pulmonary calcification, particularly the miliary type, is frequently associated with histoplasmin sensitivity. Likewise, on the basis of studies by Christie and Peterson,[23-25] Palmer,[26, 27] and Beadenkopf and associates[28, 29] it is now well established that geographical areas in the United States where the prevalence of skin test sensitivity to histoplasmin is high also show a high prevalence of pulmonary calcification among the population. It is of interest, however, that the majority of the epidemic outbreaks reported by Furcolow and Grayston[9, 10] occurred in areas of low or medium prevalence of skin test sensitivity to histoplasmin. Thus, the individuals involved in these epidemics in all likelihood had not had previous exposure to the fungus.

How man acquires histoplasmosis is not clearly understood. Although *H. capsulatum* has been isolated from a variety of animals,[30, 31] there is no evidence of animal-to-man or man-to-man spread of the disease. There is evidence of spread among animals (dogs) as shown by the studies of Prior and Cole[32] and Robinson and associates.[33] Animals chronically infected may serve, as Emmons[30] points out, in seeding the soil with the fungus.

The finding of *H. capsulatum* in the soil where animal[31] and human infections are occurring and particularly at the point source in the immediate environment of individuals who have become ill, as was the case of the E. family[1-3] and other epidemic outbreaks studied by Furcolow and Grayston[9, 10] indicates strongly that pulmonary histoplasmosis is an air-borne infection resulting from the inhalation of fungus spores. The high frequency of pulmonary infections among laboratory personnel working with the spore phase *H. capsulatum* supports this view.[34-36] Our study of the silo soil and those of Grayston and Furcolow[10] show that *H. capsulatum* may persist in the soil for several years.

The prevalence of skin test sensitivity to histoplasmin in the United States[29] and other countries[37] indicates that *H. capsulatum* is widely distributed. Assuming that a positive skin test to histoplasmin represents a previous experience with the fungus, the geographical variation in skin test sensitivity to histoplasmin in the United States may be considered reasonably a reflection of the degree of contamination or growth of *H. capsulatum* in the soil. In areas where a high prevalence of skin test sensitivity to histoplasmin exists, it has been shown that individuals have sufficient exposure to *H. capsulatum* to acquire this sensitivity at an early age.[38, 39] A recent study among Illinois residents of high school and college age reveals that 90 per cent of those living in southern Illinois react to histoplasmin in a positive manner.[40] Thus, considering the skin test with histoplasmin an epidemiological tool, it has been estimated that approximately 30 million people in the United States have had some form of infection with *H. capsulatum*.

Less than ten years ago histoplasmosis was considered a rare and generally fatal disease.[41, 42] Since this time, many cases of recovery from this disease have been reported.[43-46] These, along with the study of the E. family, those of Furcolow and Grayston[9, 10] of other epidemic outbreaks, and those concerned with prevalence to histoplasmin skin test sensitivity[23, 26, 29] strongly indicate that recovery from histoplasmosis should be considered the rule, for only rarely does it terminate as a fatal illness. The histoplasma infections in the six members of the E. family demonstrate the wide range of severity and varied manifestations of this disease, as has been emphasized in recent review articles on this subject.[46-48] They ranged from the inapparent infection without pulmonary findings to the clinically severe illness involving the general dissemination of the fungus in the host. Mr. E. represents another case who has recovered after a prolonged disseminated infection due to *H. capsulatum* without benefit of specific therapy.

Although *H. capsulatum* was not isolated in the case of E. E. and A. E., Jr., who helped their father clean the silo, their extensive pulmonary infections were undoubtedly due to this fungus. These conclusions are based on the facts

that (a) they were exposed to the silo dust at the same time and (b) they had a similar onset of illness, chest x-ray findings, significantly high C.F. antibody titers, and positive skin tests to the histoplasmin antigen. Of special significance in the case of E. E. is the conversion of the histoplasmin skin test from a negative to a positive reaction during the febrile stage of his illness. The initially positive C.F. tests, along with the positive skin tests with histoplasmin and negative tuberculin reactions, undoubtedly classify the peculiar pulmonary infiltrates in the two daughters and son who had no clinical manifestations of disease as due to *H. capsulatum*. After three years the positive skin tests are the only remaining evidence that the pulmonary infections in the E. family may have been due to the histoplasma fungus.

In the mild acute pulmonary illnesses, *H. capsulatum* usually cannot be isolated from the sputum, blood, or bone marrow, but it provokes conversion of the skin test from a negative to a positive reaction and the development of C.F. antibodies.[49-51] There is some evidence that the level of histoplasma antibody is proportional to the severity of illness.[51] C.F. antibody, as shown in the case of Mr. E. and his two sons, may persist for several months after acute symptoms subside but, with recovery, gradually disappear from the circulation. In the case of chronic active pulmonary infections C.F. antibody may persist in high titer for several years. At the terminal stage of fatal disseminated histoplasmosis, both the C.F. and skin test may be, or become, negative. In such cases *H. capsulatum* usually can be readily cultured from the blood, bone marrow, bronchial or biopsy material, or be seen on direct examination of biopsy material. Primary mucous membrane and skin lesions due to the histoplasma fungus may not provoke C.F. antibody or skin sensitization to histoplasmin, but these organisms generally can be cultured or observed in biopsy specimens. Critical illness due to other causes may also depress the skin test reaction with histoplasmin.[52] These observations must be considered when employing the C.F. technique and histoplasmin skin test as diagnostic and prognostic procedures.

Pulmonary histoplasmosis is the most frequent form of the disease and must be considered in the differential diagnosis of a variety of acute and chronic infections of the lung including acute respiratory disease, viral pneumonia,[53] other mycotic infections,[54] and tuberculosis.[55] The healed but not yet calcified fibrotic nodule may also simulate primary or metastatic lung neoplasms, lymphoma, and sarcoidosis. Careful history taking concerning the place of residence and the simultaneous use of the tuberculin, histoplasmin, coccidioidin, and blastomycin skin test antigens will aid materially in reaching a correct diagnosis.[53, 54]

Fortunately, the great majority of patients with histoplasmosis recover without specific therapy. Ethyl vanillate alone or in combination with other antifungal agents has been most frequently used in the treatment of histoplasmosis. Reports with this form of therapy indicate sufficient success to warrant further trial.[56-59] In chronic active pulmonary histoplasma infections resection of the lung has been successfully carried out.[60, 61]

SUMMARY

The changing clinical and laboratory findings in six members of the E. family who had pulmonary infections due to *H. capsulatum* have been observed over a period of three years. The father had a severe prolonged illness during which the fungus was isolated from the sputum and bone marrow. Two sons had acute, extensive but less severe pulmonary infections; the daughters and oldest son had lesser pulmonary involvement and no symptoms. Diagnosis was made by a combination of procedures including culture, C. F. test employing the yeast phase antigen, and skin tests with histoplasmin, coccidioidin, and tuberculin antigens.

With recovery, the C.F. test became negative and the skin tests with histoplasmin have been consistently positive. The father and mother also acquired and have maintained positive reactions to tuberculin since January, 1952. None have reacted to coccidioidin. The extensive disseminated infiltrates in the lungs of the father and his two sons have undergone healing by fibrosis and calcification. Calcification of the lung lesions occurred most rapidly in the youngest son and most slowly in the father. The hilar infiltrate in M. E. healed by resolution while the lung infiltrates of the other daughter and oldest son showed essentially no change.

The source of *H. capsulatum* on the farm was found to be in the silo which was cleaned by the father and his two sons approximately three weeks before the onset of their illnesses. The E. family illnesses are the first in which the source of the Histoplasma fungus which caused the infections was readily found in the immediate environment.

The epidemiology and clinical manifestations and laboratory findings in the case of the E. family have been discussed in relation to the findings of others.

We gratefully acknowledge the contributions of the following individuals to this study, Drs. R. G. Bloch and William Lester, Jr., supervised the medical care of Mr. A. E., Sr., while in the hospital. Miss Mary McCarthy, Messrs. Jason Appel and E. Russell Alexander gave valuable technical assistance in the laboratory. Dr. Charles E. Smith of the School of Public Health, the University of California, kindly supplied the coccidioidin. Histoplasmin was generously supplied by Drs. Amos Christie and J. C. Peterson of Vanderbilt University; and Dr. C. E. Roach of Eli Lilly & Company. Drs. J. W. Crosson and G. M. Bayne, Sharp & Dohme, Inc., supplied the tuberculin P.P.D. The Aureomycin and Terramycin employed in the isolation of the fungus from the soil were generously supplied, respectively, by Dr. Stanton M. Hardy, Lederle Laboratories, and Dr. Elliott R. Weyer, Chas. Pfizer & Company, Inc.

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EXPERIMENTAL STUDIES ON BACTERIAL CONTAMINATION OF BANK BLOOD

I. The Nature of "Toxicity" of Contaminated Blood

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SEVERE reactions following the transfusion of massively contaminated bank blood have been reported with increasing frequency.[1-5] The clinical picture produced by the infusion of large numbers of gram-negative bacteria into blood consists of chills and fever, beginning usually within an hour after the transfusion starts, nausea, and a rapid drop in blood pressure, with peripheral vascular collapse. Many such reactions have been fatal.[1-3]

In view of the seriousness of the reaction to bacterially contaminated blood and the widespread use of whole blood in medical therapy, the present study was undertaken to answer three main questions: (1) What conditions of storage of artificially contaminated whole blood permit the abundant growth of gram-negative bacteria? (2) What therapeutic measures can be employed to reduce the mortality rate or extend the survival time of experimental animals receiving lethal amounts of contaminated blood? (3) Is the sudden death of experimental animals following injection of blood containing large numbers of ordinarily nonpathogenic gram-negative bacteria due primarily to an overwhelming invasive infection, or to intoxication? This paper summarizes the experiments designed to answer the above questions.

METHODS AND MATERIALS

Blood.—The blood, procured in the usual fashion, was received within one to two days after collection from the donor and stored at 4° C. until used.*

Bacteria.—The two strains of gram-negative bacteria used in this investigation were tentatively identified as *Escherichia intermedium* and *Paracolobactrum intermedium.* The coliform bacillus (designated strain No. 4) was isolated from a contaminated plasma pool and the paracolon bacillus (strain No. 2) from a bottle of blood believed to have been associated with a serious transfusion reaction. The in vitro antibiotic sensitivity† of each organism is as follows: the coliform organisms were sensitive to 2.5 μg per milliliter of streptomycin, chlortetracycline, oxytetracycline and chloramphenicol; the paracolon organisms were sensitive to 10 μg per milliliter of streptomycin, 5.0 μg of chlortetracycline, 2.5 μg of chloramphenicol and were not inhibited by 10 μg per milliliter of oxytetracycline.

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†Minimum concentration of antibiotic causing complete inhibition of growth in broth for 18 hours at 37° C.

Animals.—White swiss mice weighing 12 to 18 grams were employed for determination of reactions to bacterially contaminated blood. The animals were injected intraperitoneally and closely observed for forty-eight hours. Deaths were recorded hourly for six hours and at regular intervals thereafter. Only those animals dying within forty-eight hours after the injection were recorded as deaths due to injection of the toxic material.

Preparation of Contaminated Blood.—Artificially contaminated blood was prepared by inoculating 100 ml. of whole sterile blood in rubber-stoppered vaccine bottles with 1.0 ml. of a twenty-four-hour broth culture of the test organism. The inoculated blood was incubated at room temperature for three days and thereafter refrigerated at 4° C. Standardization of the lethal dose of the contaminated blood on the LD50 basis proved impractical. Instead, the LD100 was estimated by injecting 0.1 to 0.5 ml. of "toxic" blood intraperitoneally into groups of mice, and 1 to 5 LD100 were used in the tests for therapeutic action of various agents.

Drugs.—Stock solutions of streptomycin, oxytetracycline,* chloramphenicol,† and chlortetracycline,‡ in sterile saline solution were made up fresh for each experiment and stored in the refrigerator until used.§

Preparation of Crude Endotoxin.—Esch. intermedium was grown on agar in ten Blake bottles for twenty-four hours at 37° C. The bacterial growth from each bottle was suspended in a few milliliters of distilled water and pooled. The pooled suspension was centrifuged at 3,000 r.p.m. for thirty minutes, the supernatant discarded and the sediment resuspended in 15 ml. of saline solution. This concentrated suspension contained $10^{12.5}$ viable cells per milliliter, as determined by plate count. It was twice extracted with 9 volumes of an equal mixture of absolute ether and ethyl alcohol (95 per cent) and twice with 9 volumes of acetone (C.P.) at 4° C. for twenty-four-hour periods. Following the second acetone washing, the suspension was dried to constant weight in vacuo over calcium chloride. The final produce consisted of a fluffy white powder weighing 998 mg. The crude endotoxin preparation was diluted with sterile saline solution immediately before use and titrated by inoculating graded doses intraperitoneally into groups of mice prior to each separate experiment. Approximately 1 LD100 was used in all tests.

Preparation of Antibacterial Serum.—Antiserum against *Esch. intermedium* was prepared by repeated intravenous injection of a formalinized bacterial suspension into a rabbit. The agglutinin titer of this antiserum was 1:1,280.

Definitions.—In this paper, the word "toxic" is used synonomously with the word "lethal" and serves to describe materials which produce the clinical signs described below and death of the mice within forty-eight hours after injection.

Effects of the Injection of Contaminated Blood Into Mice.—Mice injected intraperitoneally with blood containing 10^8 to 10^9 viable coliform or paracolon bacteria exhibit signs similar to those seen upon intravenous injection of certain viral or rickettsial toxins.[6-9] The first signs usually appeared approximately four to six hours after the injection and consisted of lethargy, ruffled fur, and a hunched posture. Later the breathing became labored. Bloody diarrhea appeared in some animals. A few animals died as early as five hours after the injection but the majority succumbed between twelve and eighteen hours. A few of the animals, particularly those which survived as long as thirty-six hours, manifested signs of neurological involvement, such as convulsions, incoordination, or paralysis.

EXPERIMENTAL RESULTS

The Multiplication of Paracolon Bacteria in Whole Blood at 4° C. and the Effect of Short Exposure to Room Temperature.—In order to determine the

*The trade-mark of Chas. Pfizer & Co., Inc., for the antibiotic, oxytetracycline, is Terramycin.

†The trade-mark of Parke, Davis & Co., for the antibiotic, chloramphenicol, is Chloromycetin.

‡The trade-mark of Lederle Laboratories Division for the antibiotic, chlortetracycline, is Aureomycin.

§All antibiotics were supplied by Dr. Frederick C. Fink of Chas. Pfizer & Co., Inc. A suspension of cortisone acetate was supplied by Dr. E. Alpert of Merck and Company, Inc., and dilutions of the drug in saline solution were made immediately before use.

effects of the size of the inoculum on the rate of multiplication of paracolon bacteria at low temperature, one hundred fold dilutions of a twenty-four-hour broth culture of *P. intermedium* were inoculated into 100 ml. amounts of whole sterile blood (three days old) in rubber-stoppered vaccine bottles. The inoculated blood remained at room temperature for one hour and then was placed in a refrigerator at 4° C. On the sixth and fourteenth days after inoculation, the blood was removed from the refrigerator and left at room

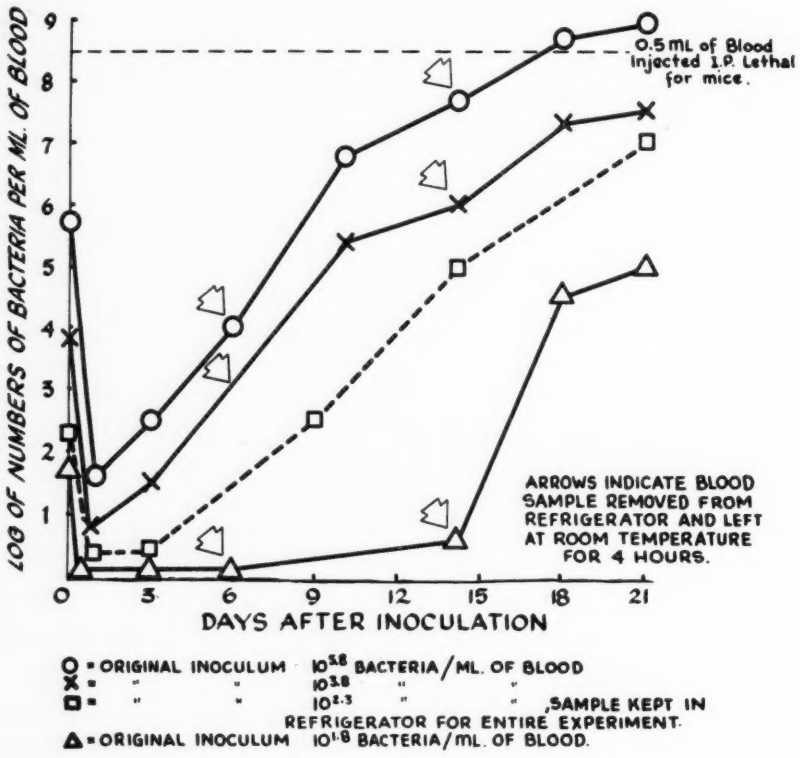

Fig. 1.—The growth rate versus inoculum size of paracolon bacteria in whole blood at 4° C. Blood three days old.

temperature for four hours. This was done to determine the effects of short periods of rising temperature, such as may occur with interruptions of refrigeration, on the rate of bacterial multiplication. At various intervals, aliquots of each blood sample were plated to determine the number of viable cells present. At the same time, a portion of the blood was injected into groups of mice to determine its toxic effects.

The results of a representative experiment, illustrated in Fig. 1, confirmed the expectation that the numbers of viable cells would drop sharply within the first few hours after inoculation, since many fresh bloods are known to contain substances inhibitory or lethal for a wide range of bacteria.[10] Of greater significance was the finding that regardless of the number of bacteria

inoculated or destroyed within the first few hours, a few cells regularly persisted and eventually multiplied. Even with inocula of approximately 100 bacteria per milliliter of blood, some cells survived in the refrigerated blood for periods up to one week before detectable multiplication occurred. This greatly extended lag period of small inocula of paracolon bacteria in refrigerated blood has been observed repeatedly in the course of the present investigation. Short periods of incubation of the blood at room temperature on the sixth and fourteenth days resulted only in a small increase in the rate of bacterial multiplication over that kept continuously at 4° C., with the possible exception of the smallest inoculum. The rate of multiplication under these conditions appeared to be roughly correlated with the initial numbers of bacteria surviving the first few hours after inoculation, the number of survivors being directly proportional to the original inoculum.

The injection of contaminated blood produced no deaths of mice until the viable bacterial population reached a certain critical concentration of 10^8 to 10^9 organisms per milliliter. Subsequent experiments showed that the toxicity of the contaminated blood was proportional to the number of *viable* cells injected and that even a tenfold dilution of the critical concentration was sufficient to abolish all clinical manifestations of toxicity. The reduction of numbers of viable bacteria in the contaminated blood by the addition of antibiotics also abolished the toxicity. Attempts to separate a soluble toxinlike material from the blood by Seitz filtration were unsuccessful. As the toxicity of the contaminated blood for mice seemed to be closely related to the number of viable bacteria injected, it was desirable to determine whether the administration of antibiotics after the injection of such blood could reduce the mortality rate or extend the survival time of the treated animals.

The Effects of Antibiotics.—One to five LD100 of toxic blood was injected intraperitoneally into mice in groups of thirty. At various intervals thereafter antibiotics singly or in combinations were administered by the same route to groups of six mice. Table I shows that, with the exception of oxytetracycline against the coliform organisms, the antibiotics were capable of reducing the mortality rate significantly when given up to one hour after the injection of the contaminated blood. Combinations of streptomycin with chlortetracycline, oxytetracycline, or chloramphenicol, in dosages which singly were ineffective, protected all the animals. In addition, there was a noticeable sparing action of the antibiotics. Animals that received therapy up to six hours after the blood injection generally lived twice as long as the unprotected controls. When the drugs were administered subcutaneously, however, much of the protective effects were lost, although the survival time was still prolonged. For example, combinations of streptomycin and chlortetracycline administered intraperitoneally one hour after the injection of blood containing 10^9 viable coliform bacteria, protected all the animals, while the same combination, injected subcutaneously, protected only 65 per cent. This reduced protection is probably related to the slower adsorption of the drugs from the

TABLE I. THE EFFECT OF ANTIBIOTIC THERAPY FOLLOWING INJECTION OF CONTAMINATED BLOOD INTO MICE

NUMBER AND KIND OF BACTERIA IN BLOOD INJECTED	ANTIBIOTIC GIVEN* KIND AND DOSE	HOURS AFTER INJECTION OF BLOOD	NUMBER OF SURVIVORS† OUT OF 6 MICE INJECTED
10^8 Paracolon bacteria	Streptomycin 2,000 μg	0	2
	Streptomycin 2,000 μg	1	3
	Streptomycin 2,000 μg	3	0
	Streptomycin 2,000 μg	6	0
	Chlortetracycline 2,000 μg	0	6
	Chlortetracycline 2,000 μg	1	6
	Chlortetracycline 2,000 μg	3	4
	Chlortetracycline 2,000 μg	6	0
	Oxytetracycline 2,000 μg	0	3
	Oxytetracycline 2,000 μg	1	2
	Oxytetracycline 2,000 μg	3	0
	Oxytetracycline 2,000 μg	6	0
	Chloramphenicol 2,000 μg	0	3
	Chloramphenicol 2,000 μg	1	0
	Chloramphenicol 2,000 μg	3	0
	Chloramphenicol 2,000 μg	6	0
	None		0
10^9 Coliform bacteria	Streptomycin 2,000 μg	0	5
	Streptomycin 2,000 μg	1	1
	Streptomycin 2,000 μg	3	0
	Streptomycin 2,000 μg	6	0
	Chlortetracycline 2,000 μg	0	2
	Chlortetracycline 2,000 μg	1	0
	Chlortetracycline 2,000 μg	3	0
	Chlortetracycline 2,000 μg	6	0
	Oxytetracycline 2,000 μg	0	0
	Oxytetracycline 2,000 μg	1	0
	Oxytetracycline 2,000 μg	3	0
	Oxytetracycline 2,000 μg	6	0
	Chloramphenicol 2,000 μg	0	4
	Chloramphenicol 2,000 μg	1	3
	Chloramphenicol 2,000 μg	3	2
	Chloramphenicol 2,000 μg	6	0
	None		0
10^9 Coliform bacteria	Streptomycin 1,000 μg + Chlortetracycline 400 μg	1	6
	Streptomycin 1,000 μg + Oxytetracycline 400 μg	1	6
	Streptomycin 1,000 μg + Chloramphenicol 400 μg	1	6

*All antibiotics injected intraperitoneally.
†Animals surviving forty-eight hours after injection of contaminated blood.

tissues and, hence, inability to prevent invasion of the bloodstream by the injected bacteria from the abdominal cavity. Studies on the bacteremia caused by these organisms will be reported at a later date.

The Effects of Cortisone.—Cortisone or ACTH may produce symptomatic improvement in various experimental or natural infections without significantly altering the outcome of the disease.[11-13] These two agents can suppress the febrile response and the inflammatory reaction to many noxious agents. Thus, Duffy and Morgan[14] and Germuth and associates[15] reported that pretreatment with either cortisone or ACTH protected rabbits against the febrile and toxic effects of Shigella and Salmonella endotoxins. Boyer and Chedid[16] found that prior treatment with cortisone protected mice against the lethal effects of the endotoxins of Salmonella, Shigella, and coliform bacteria but not against the experimental infections by the same organisms. It might, therefore, be assumed that these hormones can occasionally exert a protective effect when damage to the host is due to a toxic substance, but are probably useless or harmful when death is due to the invasive properties of the infectious microorganism.

TABLE II. THE EFFECTS OF PRETREATMENT OF MICE WITH CORTISONE OR ANTIBACTERIAL SERUM ON THE TOXICITY OF CONTAMINATED BLOOD AND CRUDE ENDOTOXINS

MICE PRETREATED WITH:	MICE INJECTED WITH:	NUMBER OF MICE SURVIVING FOR 48 HOURS‖
Cortisone*	Contaminated blood‡	2/20
Cortisone	Crude endotoxin§	1/20
Antiserum†	Contaminated blood	8/10
Antiserum	Crude endotoxin	1/10
—	Contaminated blood	1/10
—	Crude endotoxin	1/10
—	Cortisone	20/20

*Cortisone, 5.0 mg., injected subcutaneously one hour before contaminated blood or endotoxin.

†Antibacterial serum (agglutinin titer = 1:1,280) 0.5 ml., injected intraperitoneally four hours prior to blood or endotoxin.

‡Blood, 0.5 ml., containing 10^9 viable coliform bacteria injected intraperitoneally.

§Crude endotoxin, 2.0 mg., injected intraperitoneally. This amount of endotoxin equivalent to approximately 10^{11} bacteria.

‖Number of survivors per number of mice injected.

With the hypothesis that a protective effect by cortisone against the toxicity of living coliform bacteria or the endotoxins derived from the same cells might indicate the mode of death following injection of lethal numbers of living bacteria, the following experiments were made: groups of mice were given three equal injections of cortisone in total doses ranging from 0.3 to 10.0 mg. per mouse from twenty-four hours before to six hours after the injection of blood containing 10^9 viable coliform bacteria. There were no observable differences in the mortality rate or the survival time between the cortisone-treated and the untreated controls. In other experiments, mice were given total doses of cortisone ranging from 1.2 to 5.0 mg. from one to forty-eight hours prior to the injection of contaminated blood or crude endotoxin preparation. No protective effects of cortisone were noted under these conditions, either.

In contrast to the results obtained with cortisone, antiserum against the coliform bacteria conferred protection against the toxicity of the living cells

but not against the crude endotoxins. The results of representative experiments with cortisone and with antiserum are compared in Table II. It should be noted that 10^9 living coliform bacteria or 2.0 mg. of crude endotoxin preparation each constitute approximately 1 LD100 but that the latter is derived from approximately 10^{11} bacteria.

The Comparative Toxicity of Living, Heat-Killed, and Ultrasonic Irradiated Coliform Bacteria Grown in Broth.—Since the toxicity of contaminated blood for mice appeared closely related to the number of viable bacteria injected, it was of interest to determine the toxicity of dead bacteria, or living bacteria grown in media other than blood.

Accordingly, coliform bacteria were grown in Proteose peptone No. 3 broth (Difco) for twenty-four hours at 37° C., centrifuged, and washed three times with sterile saline solution. The sedimented cells were resuspended in one-tenth the original volume in saline solution and divided into two equal portions. One portion was placed in the refrigerator without further treatment, while the second portion was heated in a water bath at 75° C. for one hour to kill the cells. At the conclusion of the heating period, tenfold dilutions of both suspensions were injected into groups of mice. In another experiment, the bacteria were grown and concentrated as above and then exposed to ultrasonic irradiation* for one hour in order to obtain an endotoxin preparation for comparison with the heat-killed cells.

TABLE III. THE TOXICITY OF BROTH-GROWN LIVING, HEAT-KILLED AND ULTRASONICALLY DISRUPTED COLIFORM BACTERIA FOR MICE. ALL SUSPENSIONS INJECTED INTRAPERITONEALLY

LIVING CELLS		HEAT-KILLED† CELLS		ULTRASONICALLY IRRADIATED CELLS	
NO. OF CELLS INJECTED	NO. OF DEATHS*	NO. OF CELLS INJECTED	NO. OF DEATHS	NO. OF CELLS‡ INJECTED	NO. OF DEATHS
10^{10}	5/5	10^{10}	4/5	10^{10}	4/5
10^9	5/5	10^9	0/5	10^9	0/5
10^8	0/5	10^8	0/5	10^8	0/5

*Number of deaths per number of animals injected.

†Cells heated in a water bath at 75° C. for one hour.

‡Original suspension before irradiation contained 10^{10} viable bacteria per milliliter after irradiation $<10^5$ viable bacteria per milliliter.

Table III shows that living coliform bacteria grown in broth were as toxic for mice as those grown in blood. A concentration (10^9) of living cells able to kill all the animals within eight hours was without noticeable effect on the animals in 48 hours, when the organisms were killed by mild heating, or when the cells were disrupted by ultrasonic irradiations. A tenfold increase in the amount injected of either of the latter two suspensions, however, was able to kill most of the animals. It is not likely that the mild heating procedure employed inactivated the endotoxins of this organism since the endotoxins of other gram-negative bacteria are known to resist much higher temperatures for even longer periods.[17] Moreover, endotoxin preparations obtained through disruption of the cells by ultrasonic irradiation showed the same level of toxicity.

*Fifteen milliliters of a saline suspension of coliform bacteria was irradiated for one hour in a "Hypersonic," Model Bu 301 apparatus manufactured by the Brush Developing Co., Cleveland, Ohio.

DISCUSSION

Pittman[18] has pointed out that the entrance of microorganisms into bank blood is probably determined by chance, but the organisms which are able to survive and multiply are selected chiefly on the basis of the following characteristics: their ability to resist the lethal action of the blood, their ability to utilize the available nutrients present, and their ability to multiply readily at a low temperature. With respect to the first point, it is often assumed that the well-known lethal characteristics of fresh blood will eradicate the few chance contaminants. Braude,[10] however, has recently re-emphasized that there is a vast difference between different bloods to eliminate a particular species of bacteria. Gram-positive organisms are most easily eliminated, while a significant number of gram-negative species can survive and multiply readily in fresh refrigerated blood. It is these latter types of bacteria which have been responsible for the majority of severe or fatal transfusion reactions. Most of the fresh bloods (up to one week old) used in this study were able to reduce the viable count of large inocula of gram-negative bacteria to low levels within a few hours, but usually not all the cells were destroyed. Even with inocula of approximately 100 bacteria per milliliter of blood, a few organisms were able to survive in the refrigerated blood for periods up to one week before obvious multiplication began.

In some of the transfusion reactions reported, attention has been called to the presence of "massive" numbers of bacteria in the contaminated blood, that is, presumably of the order of 10^8 bacteria per milliliter or more.[1, 4] The question arises whether these bacteria were originally introduced into the bank blood in large numbers, or whether the heavy contamination reflected the ability of a few organisms to multiply freely under the conditions of storage. According to the available evidence, the latter alternative appears more likely.* For example, an examination of 1,697 fresh bank bloods by Braude and his colleagues revealed contamination in thirty-eight.[3] The average bacterial count of these contaminated blood banks was fourteen bacteria or less per milliliter. In addition, Braude has shown that many species of gram-negative bacteria multiply readily from small inocula at temperatures from 4° C. to 8° C., particularly if these species were originally isolated at low temperatures.[10] While these "cryophiles" multiplied readily in the cold, a temperature of 27° C. favored the most rapid growth. One might expect, therefore, that leaving contaminated blood at room temperature for short periods on different occasions would speed up the rate of bacterial multiplication, but such was not the case here. There was no significant difference in the multiplication rate of paracolon bacteria in blood exposed to short periods of incubation at room temperature over those kept in the refrigerator during the entire experiment. Instead, the rate of multiplication appeared to be correlated with the number of bacteria surviving the first

*While the data shown in Fig. 1 might indicate that relatively large numbers of paracolon bacteria must be inoculated in order to obtain rapid growth in refrigerated blood, it appears unlikely that massive contamination is necessary for other species of gram-negative bacteria involved in transfusion reactions. For example, Braude has shown that some strains of *achromobacter* and *pseudomonas* isolated from blood bank rooms grew rapidly in blood at 4° C. from inocula of 10^2 to 10^4 bacteria (personal communication).

few hours after inoculation and the number of survivors was directly proportional to the original number of bacteria inoculated. More prolonged exposure to temperatures between 20° C. and 30° C., nevertheless, is likely to accelerate rates of growth of contaminants.

The sudden death in mice following the injection of blood containing living gram-negative bacteria pointed more to an "intoxication" than to an invasive infection, but attempts to separate a soluble toxinlike material from the contaminated blood were unsuccessful. On the other hand, various antibiotics administered up to one hour after the injection of the contaminated blood were able to reduce the mortality rate and extend the survival time significantly. The protective effects of antibiotic therapy, coupled with the finding that antibacterial serum protected mice against lethal numbers of living bacteria but not against crude endotoxin preparations, pointed to the viable cell as an important component of the acute reaction. However, blood was toxic for mice only when it contained between 10^8 to 10^9 viable bacteria per milliliter. This number of living organisms was only ten to 100 times smaller than the minimum lethal number of heat-killed cells or the equivalent of crude endotoxin preparations. Therefore, the sudden death of mice following the injection of blood containing large numbers of living organisms is most likely due to an intoxication from the bacterial endotoxins. While an organism that required inoculation of 10^8 to 10^9 living cells to cause death of a given host would not ordinarily be considered invasive, that is, capable of establishment and multiplication within the host tissues, it is not unreasonable to assume in this case that a ten- to one-hundred-fold multiplication may occur in vivo and that this number of bacteria then constitutes a lethal dose of endotoxin. Antibiotics or antibacterial serum may afford protection to mice against death from the endotoxins by preventing the injected bacteria from reaching the critical number of cells.

Reports on the use of cortisone and ACTH in experimental and natural infections are numerous and often conflicting.[11, 14, 15, 19] In this study, cortisone given in a wide range of dosages prior to, or up to six hours after, the injection of blood containing lethal numbers of living gram-negative bacteria or crude endotoxin preparations had no effect in reducing the mortality rate or extending the survival time of the treated animals. These results are in general agreement with those of other workers who have shown that while ACTH or cortisone may give symptomatic improvement in many infections, neither has any significant effect on the ultimate outcome.[12, 13] It has been reported, however, that prior treatment with cortisone protected rabbits against a lethal infection with a hemolytic staphylococcus[14] and mice against the endotoxins of gram-negative enteric bacteria.[15] For the most part, the available data are not sufficient for an adequate comparison of the many variables involved, such as animal species, kind and strain of microorganism, toxic factor(s), dosage of hormones, and its duration of action. Experiments dealing with these variables are currently under study in this laboratory.

SUMMARY

A strain of paracolon bacteria isolated from banked blood believed to have been associated with a serious transfusion reaction multiplied readily from small inocula in whole human blood stored at 4° C. Fresh refrigerated blood reduced the viable bacterial count to low levels within a few hours after inoculation, but some cells usually survived and eventually multiplied. With inocula of approximately 100 cells per milliliter of blood, the lag period was extended up to one week before multiplication began. The multiplication rate of paracolon bacteria in refrigerated blood was affected only slightly by short interruptions of refrigeration.

When artificially contaminated blood containing sufficiently large numbers of viable paracolon or coliform organisms was injected into mice, death usually resulted in from six to eighteen hours. Antibiotics or specific antibacterial serum protected the animals against the toxicity of contaminated blood. Antibacterial serum gave no protection against crude endotoxin preparations obtained from the homologous organism. Cortisone in a wide range of doses did not protect mice against the bacterial endotoxins, or against the viable organisms in the contaminated blood.

The pathogenesis of the toxicity of contaminated blood for mice was discussed from the standpoint of invasive infection of the contaminant versus intoxication due to bacterial endotoxins. The conclusion was reached that the endotoxins are primarily responsible for the acute reaction and death.

We wish to express our appreciation to Dr. A. I. Braude for his helpful suggestions and criticisms.

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ADDENDUM

After this manuscript was submitted for publication, Stevens and associates (Ann. Int. Med. **39**: 1228-1239, 1953) reported a fatal transfusion reaction due to bank blood contaminated with large numbers of pseudomonas bacteria.

DISTRIBUTION AND IN VITRO ANTIBIOTIC SUSCEPTIBILITY OF KLEBSIELLA (KAUFFMAN)

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A COMMON impression among clinicians is the belief that *Klebsiella pneumoniae* (Friedländer's bacillus) and *Aerobacter aerogenes* represent two distinct genera; the former being associated exclusively with upper respiratory tract infections, while the latter is most often associated with infections of the genitourinary tract. In an effort to find the historical reasons for this belief, Obrinsky and associates[1] suggested that, when the Friedländer's bacillus was isolated in 1882 from patients with pneumonia, it was studied primarily from the morphologic standpoint. On the other hand, Escherich, who first described *A. aerogenes* in 1886, emphasized its close similarity in biologic activity to *Escherichia coli,* which was also first described by him. Thus, from the very first, *A. aerogenes* was more intimately identified with *E. coli* than with the Friedländer's bacillus, a relationship which enjoyed general acceptance.

Extensive studies within the last quarter of a century point inexorably to the fact that while *A. aerogenes* is related, in some respects, to certain strains of *E. coli,* it is more closely related, biochemically and serologically, to the Friedländer's bacillus. In 1929, Edwards[2] reported on the morphologic and biochemical similarity of Friedländer's bacilli and cultures which, on the basis of criteria existing at the time, were classified as *A. aerogenes.* The similarity was so close that, in his opinion, there was no basis for generic differentiation of the two organisms. Other investigators[3-6] subsequently confirmed these findings and advocated the inclusion of Friedländer's bacilli and *A. aerogenes* into a single genus. The intimate immunologic relationship between these organisms has been similarly established.[7-9] These and other studies, as well as exhaustive investigations of his own, are the basis for Kauffman's[6] suggestion that a single genus be established to include both Friedländer's bacilli and *A. aerogenes* and, in view of priority, that the name Klebsiella be assigned to it. This recommendation has been adopted in our laboratory where, for the past nine months, we have been studying Klebsiella organisms from the standpoint of their relative susceptibility to antibiotics and their incidence in various clinical specimens. This report summarizes our experience to date with these organisms.

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MATERIALS AND METHODS

A. *Sources of Cultures.*—The Klebsiella strains included in the study were isolated from various clinical specimens from 305 individual hospitalized patients. Sinorespiratory tract infections furnished 21.3 per cent of the strains, infections of the genitourinary tract 62.6 per cent, and other infections (abdominal, osteomyelitis, abscesses, etc.) 16.1 per cent.

B. *Identification of Strains.*—Criteria for designating an organism as a Klebsiella strain were as follows: A gram-negative, nonmotile, aerobic bacillus which fermented lactose, adonitol, and inositol, gave positive Voges-Proskauer, citrate utilization, and urease reactions and, in addition, was indole and methyl red negative. Organisms showing this pattern were then subjected to slide agglutination tests with type-specific capsular antisera.[9] In view of the large number of Klebsiella capsular types (57) that have been identified to date[9] and the magnitude of the task coincident with the preparation of an equivalent number of specific antisera, we prepared* and used sera specific for capsular Types 1 to 9 inclusive. Within these types were included those which have been most frequently associated with sinorespiratory infections (1 to 6) as well as some types frequently associated with infections of the urinary tract (7 to 9). Strains which conformed to the biochemical pattern shown by Klebsiella, but which failed to agglutinate with any of the available antisera, were simply recorded as Klebsiella species, type undetermined.

C. *Antibiotic Susceptibility Tests.*—Procedures and criteria for definition of susceptibility have been described in detail previously.[10] Antibiotics included streptomycin, chlortetracycline, oxytetracycline, and C. O. C.,[11, 12] a mixture of chlortetracycline, oxytetracycline, and chloramphenicol in the proportion of 4:2.5:1.

RESULTS AND DISCUSSION

Fig. 1 shows the incidence of the 305 Klebsiella strains in specimens originating in various tissue sites. It is seen that approximately 47 per cent (145) of the strains were incapable of being typed with the available sera. Of this number, 60, 15.8, and 24.2 per cent were isolated from specimens originating in the urinary, sinorespiratory, and other tracts, respectively. Of the remaining 160 strains (53 per cent) representing serotypes 1 to 9, 65 per cent were associated with urinary infections, 26.2 per cent with sinorespiratory diseases, and 8.8 per cent with infections of other sites. It is of interest to note that by the use of a small number of sera representing only 16 per cent of the total number of Klebsiella serotypes demonstrated to date, it was possible to make a specific serologic identification in 53 per cent of the total number of strains studied. This would suggest that, at least in our series, the predominating Klebsiella serotypes were included in the group comprising Types 1 to 9.

The relative proportion of each of the individual types comprising this latter group is also shown in Fig. 1. Of the 160 strains which could be agglutinated with specific sera, Type 7 occurred most often (38.8 per cent) and for the most part was associated with the urinary tract. No strains of this type were isolated from sinorespiratory specimens, although a small number (10 per cent) were associated with other anatomic sites. Type 4, comprising 5.7 per cent of the strains, was uniformly isolated from sinorespiratory specimens. Types 5 and 6 were not encountered. Types 1, 2, 3, 8, and 9, repre-

*The cultures and procedures used in the preparation of specific capsular antiserum were very kindly supplied by Dr. P. R. Edwards, Communicable Disease Center, Public Health Service, Atlanta, Ga.

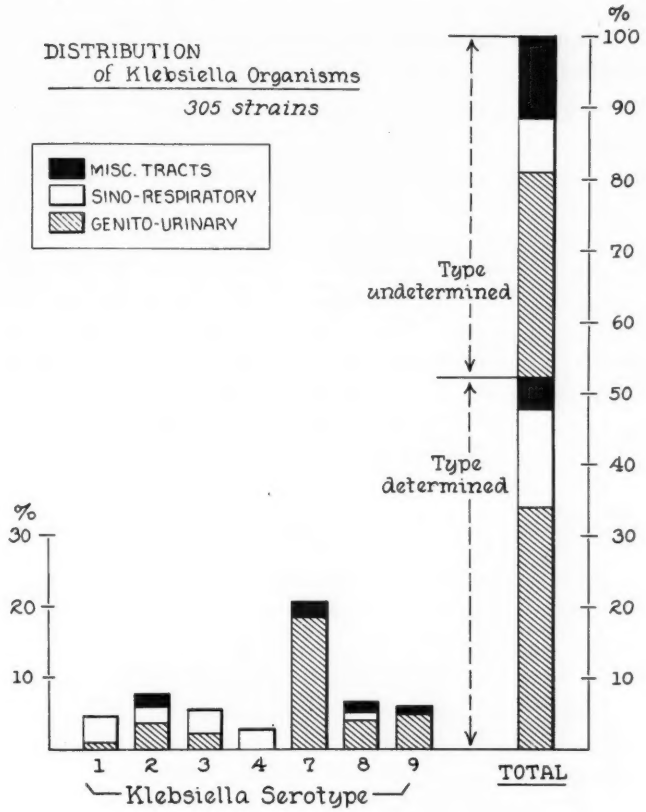
DISTRIBUTION
of Klebsiella Organisms
305 strains
MISC. TRACTS
SINO-RESPIRATORY
GENITO-URINARY
%
100
90
80
70
60
50
40
30
20
10
Type undetermined
Type determined
1
2
3
4
7
8
9
TOTAL
Klebsiella Serotype

Fig. 1.

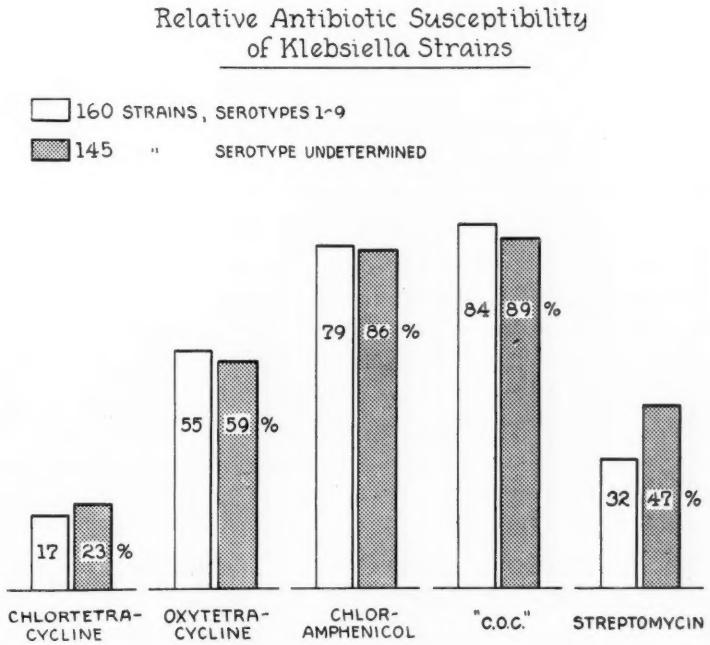
Relative Antibiotic Susceptibility
of Klebsiella Strains
160 STRAINS, SEROTYPES 1-9
145 " SEROTYPE UNDETERMINED
17
23 %
55
59 %
79
86 %
84
89 %
32
47 %
CHLORTETRA-CYCLINE
OXYTETRA-CYCLINE
CHLOR-AMPHENICOL
"C.O.C."
STREPTOMYCIN

Fig. 2.

senting 55.5 per cent of all strains, showed no specific localization in any given site. Of particular interest is the finding that of the 60 strains (Types 1 to 4 inclusive) which were formerly designated Friedländer's bacilli Types A to D and considered to be primarily pulmonary pathogens, 26, or 43.5 per cent, were isolated from specimens which were of nonrespiratory origin. This is in agreement with the experience of Lemke and Gates,[13] who reported a 45.5 per cent incidence of these types in nonrespiratory sources.

Fig. 2 shows the relative susceptibility of our Klebsiella strains to chlortetracycline, oxytetracycline, chloramphenicol, streptomycin, and the C. O. C. combination. It is seen that, except in the case of streptomycin, there is no significant difference in incidence of susceptibility of the typed and untyped strains to the various antibiotics. However, significant differences in activity of each of the agents is apparent. C. O. C. and chloramphenicol are most ef-

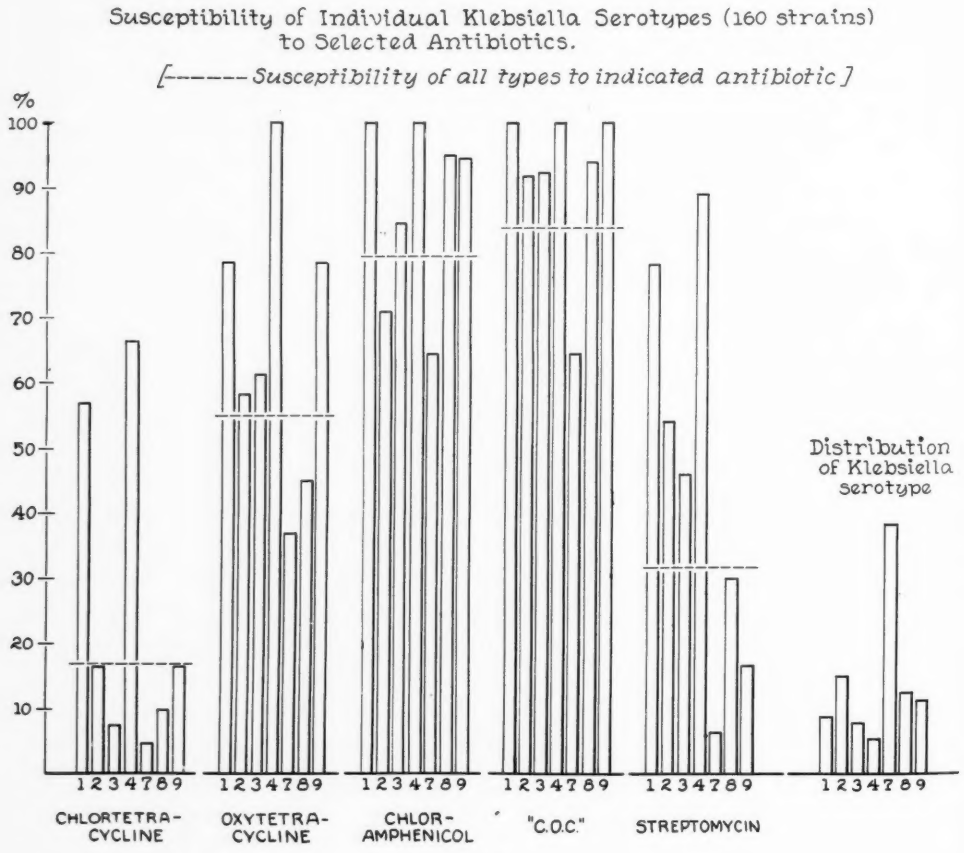

Fig. 3.

fective, with oxytetracycline, streptomycin, and chlortetracycline showing decreasing activity in the order listed. It is pointed out that while incidence of susceptibility to chloramphenicol is not significantly different from that to

C. O. C., the amount of chloramphenicol in the combination is only one-third that used as a criterion for establishing sensitivity to chloramphenicol alone.

Fig. 3 suggests the possibility of a relationship between antibiotic susceptibility and serotype of Klebsiella organisms. This figure shows the incidence of susceptibility of each serotype to the various agents compared with that of all serotypes combined.

If the level of relative antibiotic susceptibility of each serotype is gauged in relation to the number of agents toward which it shows an incidence of suceptibility above that manifested by all types combined, then it can be said that Types 1 and 4 are most susceptible, while Type 7 is least sensitive. The remaining types can be similarly classified as lying between these extremes and in the order of increasing resistance are Types 3, 2, 9, and 8. Also, if one wishes to gauge the effectiveness of the several agents on the basis of the total number of serotypes showing an increased incidence of susceptibility then, in order of diminishing activity, the agents are C. O. C., chloramphenicol, and oxytetracycline, streptomycin, and chlortetracycline. These results, however, must be interpreted with full knowledge of the limitations imposed by the total number of strains, as well as the number of each serotype, which comprise this study. It is quite conceivable that a study with a larger number of strains of more serotypes might yield results different from those presented herein. It is interesting to note, however, that results of treatment of urinary tract infections[14] and pneumonia[15] due to Klebsiella organisms with C. O. C. suggest that the superiority of this combination is not confined to in vitro conditions.

SUMMARY AND CONCLUSIONS

The results of a study of the distribution and in vitro antibiotic susceptibility of 305 Klebsiella strains are presented. Infections of the genitourinary tract furnished approximately 63 per cent of the strains, sinorespiratory tract infections 21 per cent, and other infections (abdominal, osteomyelitis, abscesses, etc.) 16 per cent. Approximately 50 per cent of all strains could be allocated to specific serotypes, including 1, 2, 3, 4, 7, 8, and 9. Types 5 and 6 were not encountered in this study.

Particularly noteworthy is the finding that of the 60 strains (Types 1 to 4) formerly designated as Friedländer's bacilli A to D and considered to be primarily pulmonary pathogens, approximately one-half were isolated from specimens which were of nonrespiratory origin.

Significant differences in incidence of susceptibility of typed and untyped strains to a given antibiotic could not be demonstrated, except in the case of streptomycin, which showed a somewhat higher degree of activity against the untyped strains. The Klebsiella organisms in this study showed the highest incidence of susceptibility to chloramphenicol and C. O. C., a combination of chlortetracycline, oxytetracycline, and chloramphenicol. In order of diminishing activity the other agents were oxytetracycline, streptomycin, and chlortetracycline.

Evidence suggesting a possible relationship between Klebsiella serotype and susceptibility to antibiotics is presented. On the basis of stipulated criteria, Types 1 and 4 are most susceptible, while Type 7 is least sensitive. The other types occupy an intermediate position. If level of effectiveness of an antibiotic agent is gauged by the total number of serotypes and number of different serotypes which show an increased incidence of susceptibility, then the most effective agent in the study was C. O. C.

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IN VITRO ACQUIRED RESISTANCE AND SENSITIVITY OF ENDAMOEBA HISTOLYTICA TO OXYTETRACYCLINE

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PREVIOUSLY Seneca and Murphy[1] reported on eleven strains of *Endamoeba histolytica* from the United States, North Africa, and Korea, concerning their sensitivity or resistance to oxytetracycline (Terramycin) when grown in the presence of oxytetracycline. Eight strains showed no change, two strains became more sensitive, and one strain became more resistant. The present report is a further study along these lines.

METHODS

Endamoeba histolytica was grown on modified Boeck-Drboklav medium of the following composition: The contents of eight eggs were emulsified in 100 ml. sterile buffered saline in a sterile flask containing glass beads. The medium was put in sterile test tubes and inspissated at 90° C., 15 pounds pressure for 90 minutes. The overlay was 5 ml. buffered saline.* Sterile rice starch (sterilized at 145° C., dry heat for 90 minutes) was added to the medium.

Eleven strains (Gloria, Casablanca, Lilly, Korea, Korea 1, Korea 6, Columbia, 722t, 200t, *E. histolytica* with *Escherichia coli* and *E. histolytica* with *Aerobacter aerogenes*) were grown in this medium for 48 hours.

Sterile intravenous oxytetracycline hydrochloride was serially diluted in buffered saline in concentrations of 10, 1, 0.5, 0.25, 0.1, and 0.05 μg/ml. Five milliliters of these concentrations of oxytetracycline in buffered saline and sterile rice starch were added to the culture medium. The culture tubes containing the antibiotic were inoculated with one of the eleven strains previously mentioned and incubated at 37° C. for 48 hours. Subcultures were made three times a week in the corresponding concentrations of oxytetracycline.

Ten micrograms per miliiliter of oxytetracycline killed all the eleven strains in the fourth subculture; therefore this concentration was abandoned. Gloria, Casablanca, and Lilly strains could not survive in 1, 0.5, and 0.25 μg/ml. concentrations after the fourteenth subculture and therefore they were serially grown in 0.1 and 0.05 μg/ml. concentrations. In these concentrations Lilly and Casablanca strains stopped growing in the thirty-fourth subculture. The other eight strains grew very well in 1, 0.5, and 0.25 μg/ml. concentrations of oxytetracycline. Columbia and Korea 1 strains, which were grown in oxytetracycline hydrochloride for 48 subcultures, were grown on normal medium in the absence of the antibiotic for 20 subcultures. Similarly Gloria and *E. histolytica* with *E. coli*, which were grown for 35 and 28 subcultures, respectively, in oxytetracycline, were grown on normal medium in the absence of the antibiotic for ten generations. These experimental strains were compared with their respective stock culture controls concerning their behavior in relation to oxytetracycline.

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*M/15 $Na_2HPO_4 \cdot 12H_2O$ (1.72 Gm. per 100 ml.): 82.0 ml.
M/15 KH_2PO_4 (0.907 Gm. per 100 ml.): 5.0 ml.
NaCl C.P. 8.5 Gm.
Distilled water q.s.: 1,000.0 ml.
Sterilize at 15 pounds pressure for 15 minutes.

RESULTS

Table I shows in a tabular form the effect of growing eleven strains of *E. histolytica* in 0.05 to 1.0 μg/ml. oxytetracycline hydrochloride and compared with their respective stock control cultures. Casablanca, Lilly and Gloria strains were grown in 0.05 to 0.10 μg/ml. Casablanca and Lilly strains in the twentieth subculture and Gloria in the thirty-fifth subculture in oxytetracycline as well as their stock control cultures were inhibited in a concentration of 125 μg/ml., indicating that there was no change in their sensitivity when grown in the presence of oxytetracycline. Strains 200t, 722t, and *E. histolytica* with *E. coli* or *A. aerogenes* were grown for 28 subcultures in 0.25

TABLE I. THE EFFECT OF GROWING ELEVEN STRAINS OF E. HISTOLYTICA IN 0.05 TO 1.0 μG/ML. OXYTETRACYCLINE HYDROCHLORIDE AND COMPARED WITH THEIR RESPECTIVE STOCK CONTROL CULTURES

	CONCENTRATION OF OXYTETRACYCLINE HYDROCHLORIDE IN MICROGRAMS PER MILLILITER							
ENDAMOEBA HISTOLYTICA STRAINS	500	250	125	62.5	31.25	15.625	7.81	Control
Casablanca*								
Subculture 20	−	−	−	+	+	+	+	+
Control	−	−	−	+	+	+	+	+
Lilly*								
Subculture 20	−	−	−	+	+	+	+	+
Control	−	−	−	+	+	+	+	+
Gloria*								
Subculture 35	−	−	−	+	+	+	+	+
Control	−	−	−	+	+	+	+	+
200t								
Subculture 28	−	−	−	+	+	+	+	+
Control	−	−	−	+	+	+	+	+
722t								
Subculture 28	−	−	−	+	+	+	+	+
Control	−	−	−	+	+	+	+	+
E. histolytica with *E. coli*								
Subculture 28	−	−	−	−	+	+	+	+
Control	−	−	−	−	+	+	+	+
E. histolytica with *A. aerogenes*								
Subculture 28	−	−	−	−	+	+	+	+
Control	−	−	−	−	+	+	+	+
Korea								
Subculture 51	−	−	−	+	+	+	+	+
Control	−	−	−	+	+	+	+	+
Korea 1								
Subculture 48	−	−	−	−	−	−	+	+
Control	−	−	−	+	+	+	+	+
Korea 6								
Subculture 48	−	−	−	−	+	+	+	+
Control	−	−	+	+	+	+	+	+
Columbia								
Subculture 48	−	−	+	+	+	+	+	+
Control	−	−	−	−	+	+	+	+

*These strains were grown in 0.05 to 0.10 μg/ml. oxytetracycline hydrochloride.

to 1.0 μg/ml. oxytetracycline. The experimental as well as the stock control cultures of 200t and 722t were inhibited in 125 μg/ml. while *E. histolytica* with *E. coli* or *A. aerogenes* were inhibited in 62.5 μg/ml., indicating that there was no change in their sensitivity to the antibiotic. Korea, Korea 1, and Korea 6 strains were grown for 51, 48, and 48 subcultures, respectively, in 0.25 to 1.0

μg/ml. concentration of oxytetracycline hydrochloride. Korea subculture 51 and its control stock culture were inhibited in 125 μg/ml. Korea 1 subculture 48 was inhibited in 15.625 μg/ml. while its stock control culture was inhibited in 125 μg/ml., indicating that it had become eight times more sensitive as compared with its stock control culture. Korea 6 subculture 48 was inhibited in 62.5 μg/ml., while its stock control culture was inhibited in 250 μg/ml., indicating that it had become four times as sensitive as its original control culture. Columbia strain was grown for 48 subcultures in 0.25 to 1.0 μg/ml. oxytetracycline hydrochloride. The experimental strain was inhibited in 250 μg/ml., while its control stock culture was inhibited in 62.5 μg/ml., indicating that it had become four times as resistant as its original stock culture.

Table II shows in a tabular form the results of growing for 10 and 20 subcultures in normal medium in the absence of oxytetracycline, the four strains of *E. histolytica* which were grown for 28 to 48 subcultures in oxytetracycline hydrochloride.

TABLE II. THE EFFECT OF GROWING E. HISTOLYTICA, COLUMBIA AND GLORIA SUBCULTURES 48 IN OXYTETRACYCLINE MEDIUM FOR 20 SUBCULTURES, AND GLORIA SUBCULTURE 35 AND E. HISTOLYTICA WITH E. COLI SUBCULTURE 28 IN OXYTETRACYCLINE MEDIUM FOR TEN SUBCULTURES, IN ANTIBIOTIC FREE MEDIUM AND COMPARED WITH THEIR STOCK CONTROL CULTURES

ENDAMOEBA HISTOLYTICA STRAINS	MICROGRAMS PER MILLILITER							
	500	250	125	62.5	31.25	15.625	7.81	Control
Columbia								
Subculture 48	–	–	–	+	+	+	+	+
Control	–	–	–	–	–	+	+	+
Korea 1								
Subculture 48	–	–	–	+	+	+	+	+
Control	–	–	–	+	+	+	+	+
Gloria								
Subculture 35	–	–	–	–	+	+	+	+
Control	–	–	–	–	+	+	+	+
E. histolytica with *E. coli*								
Subculture 28	–	–	–	–	+	+	+	+
Control	–	–	–	–	+	+	+	+

The experimental Columbia strain subculture 48 was inhibited in 125 μg/ml. while its stock control culture was inhibited in 31.25 μg/ml., indicating that the original 4:1 resistance was still maintained. Korea 1, which had become eight times as sensitive in its forty-eighth subculture in oxytetracycline, reverted back to normal sensitivity because the experimental as well as the control were inhibited in 125 μg/ml. On the other hand, Gloria and *E. histolytica* with *E. coli,* which showed no change in sensitivity when grown in the presence of oxytetracycline, again showed no change when subcultured in the absence of the antibiotic. Both the experimental and the stock control cultures were inhibited in 62.5 μg/ml.

DISCUSSION

Oxytetracycline hydrochloride in 10 μg/ml. inhibited the growth of eleven strains of *E. histolytica* in the fourth subculture. In 1 μg/ml., 0.5 μg/ml. and 0.25 μg/ml. it inhibited the growth of three strains (Gloria, Casablanca, and

Lilly) in the fourteenth subculture. In concentrations of 0.1 to 0.05 μg/ml. it inhibited the growth of two strains (Casablanca and Lilly) in the thirty-fourth subculture.

In the twenty-eighth subculture in the presence of 0.25 to 1.0 μg/ml. oxytetracycline hydrochloride, strains 200t, 722t, *E. histolytica* with *E. coli,* and *E. histolytica* with *A. aerogenes* showed no increase or decrease in sensitivity to oxytetracycline. Gloria strain in the thirty-fifth and Korea strain in the fifty-first subcultures also showed no change in sensitivity to oxytetracycline. Korea 1 strain in subculture 48 was inhibited in 15.625 μg/ml., while its stock control was inhibited in 125 μg/ml., and Korea 6 strain subculture 48 was inhibited in 62.5 μg/ml. and its stock culture in 250 μg/ml., showing that these two strains had definitely become 8 and 4 times more sensitive to oxytetracycline. Columbia strain in the forty-eighth subculture in oxytetracycline hydrochloride became four times more resistant to the antibiotic, since it was inhibited in 250 μg/ml. oxytetracycline while its stock control culture was inhibited in 62.5 μg/ml. (Table II).

The increased resistance to oxytetracycline with the Columbia strain was fixed and not reversible because upon growing the experimentally induced resistant Columbia strain for 20 subcultures in normal medium in the absence of oxytetracycline, the 4:1 proportion of increased resistance was maintained (125 μg/ml.:31.25 μg/ml.). Korea 1 strain, which had become eight times more sensitive to oxytetracycline, showed a reversal of sensitivity back to normal upon growing for 10 subcultures in normal medium in the absence of the antibiotic. On the other hand, strains Gloria and *E. histolytica* with *E. coli,* which had shown no change in sensitivity, also manifested no change when subcultured in normal medium in the absence of the antibiotic.

SUMMARY AND CONCLUSION

Eleven strains of *E. histolytica* were grown from 28 to 51 subcultures in the presence of 0.05 to 1.0 μg/ml. oxytetracycline hydrochloride. Eight strains showed no change, two strains became more sensitive and one strain became more resistant.

The sensitive strain upon subculturing in normal medium for 10 subcultures reverted back to normal sensitivity while the resistant strain maintained its resistance even after 20 subcultures in the normal medium in the absence of oxytetracycline hydrochloride.

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THE SURVIVAL OF HUMAN ERYTHROCYTES ESTIMATED BY MEANS OF CELLS TAGGED WITH RADIOACTIVE CHROMIUM: A STUDY OF THE NORMAL STATE

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THE determination of the average survival time of erythrocytes has extended considerably the understanding of the mechanism responsible for the production of various anemias. The method of differential agglutination of red blood cells, as described by Ashby,[1, 2] has provided a means of determining the erythrocytic survival which has been widely applied to clinical investigation. The average human erythrocytic survival as measured by this method is 120 ± 10 days. Similar results have been obtained using red blood cells tagged with an isotope of nitrogen[4] (N^{15}) or of iron[5] (Fe^{59}). The application of the differential agglutination technique has been expanded with the use of anti-M and anti-N sera.[6, 7] In addition, a method dependent upon group specific hemolysin, rather than agglutinin, has been reported.[8] Young and co-workers[9] have discussed in detail the reliability of the differential agglutination technique.

There are inherent shortcomings in the above methods. With the exception of the method of Shemin and Rittenburg,[4] which is dependent upon N^{15} tagged glycine, the above methods require the transfusion of red blood cells into a second individual with subsequent appraisal of the survival within that recipient. Accordingly, extracorpuscular factors contributing to a hemolytic state can be evaluated by the transfusion of identifiable normal red cells into the suspected recipient; the identification of intracorpuscular factors must depend on the transfusion of the suspected cells into a recipient considered to be hematologically normal. Under the latter circumstances the recipients should be controlled by the prior demonstration that normal cells remain for the expected period of time within their circulation. Studies requiring more than one homotransfusion into one individual are always subject to the possibility of minor incompatibilities[3, 10, 11] which tend to hasten the dissipation of the transfused cells, irrespective of the hemolytic state being appraised. Thus, the differential agglutination method is potentially hampered by the necessity of having the proper immunologic make-up of the donor's and recipient's blood, by the possible increased destruction via minor incompatibilities, and by the difficulties of securing blood when known incompatibilities develop or exist. In addition, the transfusion of blood into a recipient is always associated with a risk of the transmission of viral hepatitis.

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The previously mentioned shortcomings have not prevented the collection of a considerable amount of fundamental data dealing with various anemias by the differential agglutination method.[3] This would indicate that these shortcomings have usually been overcome. There is one deficiency of the differential agglutination method which cannot be overcome, namely, the inability of this approach to determine the survival characteristics of an individual's erythrocytes within the same individual's circulation. The optimal arrangement for determining the existence of a hemolytic state is thus not possible.

Two methods utilizing radioactive isotopes have been suggested for the purpose of estimating the survival of red blood cells within their natural habitat. The first one of these utilizes the turnover rate of plasma and red cell iron, and the fraction of red cell iron renewed per day.[12] The turnover rate of radioactive iron in these studies on normal subjects corresponded with a normal red cell survival of 120 days. This method has been used to evaluate the rate of turnover of iron, and accordingly of red cells in various pathologic states. The second method utilizes the tagging of red cells with radioactive chromium in vitro and the measurement of the disappearance of the radioactivity within the recipient's circulating blood as a measurement of the disappearance of the tagged red cells. This approach appeared justified after the demonstration by Gray and Sterling[13] that red blood cells can be tagged with radioactive chromium. Up to the present, techniques utilizing Cr^{51} tagging of red cells[14, 15] have yielded results considered to be approximately 50 to 75 per cent of that expected by the other methods mentioned above.

In the present communication we wish to present a method for testing the survival of red blood cells in normal individuals utilizing the in vitro tagging of erythrocytes with Cr^{51}, which yields results comparable to the Ashby differential agglutination technique when the isotope method is used in parallel with the Ashby technique, as well as when the radioactive isotope method is used alone by utilizing the individual's own cells for the purpose of tagging.

MATERIAL AND METHODS

The normal individuals in the present study consisted of 10 young physicians and technicians. All were normal, nonanemic, and only one (Case 1) had received blood previously. There were two women (Cases 3 and 4) and 8 men. In the 5 instances where two techniques of cell survival were used, the differential agglutination method described by Young and co-workers[9] was used in conjunction with the radioactive chromium method.

The erythrocytes were tagged in vitro with sodium radiochromate ($Na_2Cr^{51}O_4$). Fifty to sixty-five milliliters of whole blood were drawn from an antecubital vein without stasis into a sterile heparinized syringe or drawn off aseptically from a pint of whole blood being used to measure the red blood cell survival by the Ashby technique. The blood was transferred into a sterile 100 ml. rubber-capped bottle of the multiple dose vaccine type. The blood was centrifuged; the supernatant plasma was removed and set aside in the refrigerator. The erythrocytes were resuspended in normal saline containing 200 to 300 microcuries of Cr^{51}, added as sterile isotonic $Na_2Cr^{51}O_4$.* This red blood cell suspension was incubated at 37° C. for one hour while constantly shaken. The supernatant saline was removed after centrifugation and the cells were washed three times with 50 ml. of cold

*Abbott Laboratories, Radioactive Pharmaceutical Division, Oak Ridge, Tenn.

normal saline. As part of the first saline wash the cell suspension was filtered through a sterile disposable blood filter.* Following the third saline wash the supernatant saline was removed and the cells were resuspended in the original plasma. An aliquot of this reconstituted blood was taken for determining the radioactive count when a blood volume measurement was planned as an initial part of the cell survival study. The remainder of the reconstituted blood was injected intravenously from a calibrated syringe. When a blood volume determination was conducted, a sample was withdrawn after three hours from the opposite antecubital vein. The initial sample used in the study of the erythrocytic survival was withdrawn twenty-four hours after the injection of the tagged red cells. When the red cells tagged with radiochromium were derived from a pint of blood used to measure the red cell survival by the Ashby method, the samples for both survival studies were obtained from a single venous blood sample collected without stasis.

The Ashby technique was instituted in the normal subjects by removing one pint of blood and immediately replacing it with a pint of blood containing nonagglutinable cells. The compatibility of the heterotype blood was established by the regular cross-matching procedure and by the use of the indirect Coombs' test as applied to the donor's erythrocytes and the recipient's serum. The blood types of the donors and recipients used in the Ashby technique were as listed in Table I. The blood was sampled daily for three to five

TABLE I. BLOOD TYPES OF DONORS AND RECIPIENTS USED IN THE ASHBY METHOD. TYPE D IS THE SAME AS Rh_0

CASE	TYPE DONOR BLOOD	TYPE RECIPIENT BLOOD
1	O D–	B D–
2	O D+	A D+
3	O D+	A D+
4	O D+	A D+
5	O D+ N+ M–	O D+ N+ M+

days after the injection of the tagged cells and then at weekly intervals. The volume of blood removed for this sampling was not significant in the human being as in general the quantity amounted to 10 ml. Sampling was continued until there was no significant radioactivity detected from a 4 ml. whole blood sample or until the nonagglutinable cell count had reached the base-line value. The radiochromium tagged cells could be detected within the normal individual for as long as 112 days. The Ashby differential agglutination study was conducted for 90 to 113 days.

The hematocrit reading was obtained in triplicate on each sample used in the study. Significant deviations of this measurement were not detected during the various studies thus suggesting a relatively constant red cell mass.

The radioactivity from 4 ml. of blood was detected by a gamma sensitive sodium iodide-thallium scintillating well type crystal.† The scintillating crystal and a photomultiplier tube‡ with preamplifier tubes produce pulses which can be recorded by any of the usual scaler circuits. The detecting unit used in our laboratory is shielded by approximately 500 pounds of lead in the form of machined rings. Each ring is portable and can be removed to permit servicing of the unit. The unit was designed by Mr. Leslie Demarest in the Radioisotope Unit, Minneapolis Veterans Administration Hospital, and was modified during its production locally by Mr. Robert Huffines, Consultant in Electronics at the Dallas Veterans Administration Hospital, Radioisotope Unit. This detector unit has a background of 2.4 counts per second and records 400 counts per second per microcurie of radioactive chromium (Cr^{51}).

The data accumulated in the radiochromium study were treated in such a manner as to allow comparison with the Ashby technique. The Ashby erythrocyte survival curve is an expression of the per cent survival of nonagglutinable cells at any time (t) with

*R18 Administration Set, Baxter Laboratories.
†Crystal Type 22, Harshaw Chemical Company, Cleveland, Ohio.
‡RCA, No. 5819 Photomultiplier tube.

reference to the initial nonagglutinable cell count at twenty-four hours after the transfusion of the red cells. Thus each point plotted on linear rectangular "ortho" graph paper is derived by the following formula: Per cent survival (of unagglutinable cells) at day (t) $= \frac{\text{no. of nonagglutinable RBC per c.mm. at day (t)}}{\text{no. of nonagglutinable RBC per c.mm. at 24 hours}} \times 100$. In the Cr^{51} study the radioactivity of the 4 ml. blood sample was corrected for physical decay occurring from the time of the initial sample. The survival of the radiochromium tagged red cells was considered from the data of the radioactivity present in 4 ml. of blood at any time (t) and the radioactivity present in the initial 4 ml. blood sample. Thus, each point on the curve represents the ratio of corrected radiochromium activity to the activity of the twenty-four-hour sample plotted against time in days on linear coordinate paper. "Per cent survival" of Cr^{51} tagged cells at any day (t) $= \frac{\text{c/s in 4 ml. sample at day (t) (corrected for decay)}}{\text{c/s in initial 24 hr. 4 ml. sample}} \times 100$. In actual practice all of the blood samples can be saved and counted on the same day, obviating the mathematical correction for physical decay. In utilizing the Ashby technique the following basic assumptions are made: (a) the blood volume remains fairly constant; and (b) there is a constant rate of production and destruction of erythrocytes. The counting error in measuring the radioactivity was maintained at less than 3 per cent.

RESULTS

The 5 normal individuals tested with the Ashby technique yielded curves of red cell survival of the expected normal type with extinction at 100 to 120 days. The 5 curves are given in Fig. 1. A portion of each pint of blood used in the Ashby method was treated with radiochromium and injected into the corresponding recipient. The curves of red cell survival by the second method for the same individuals are given in Fig. 2.

It is to be noted that the "survival curve" as determined by the radioactive chromium method presents a much smaller range of variation than the range of variation observed with the method of Ashby. The radiochromium curve is slightly curvilinear reaching extinction at approximately 108 to 120 days.

Each member of a second group of 5 normal men received a 50 ml. injection of their own blood (autotransfusion) which contained erythrocytes that had been tagged in vitro with radiochromium. The only difference in the blood used for this group and the former group which received donor heterotype blood (homotransfusion) tagged with Cr^{51} concerned the time between the collection of the blood and its injection. The blood used in the autotransfusion group was treated promptly and was returned to the individual's circulation within three hours as Cr^{51} tagged cells. The blood used in the group receiving a homotransfusion for both the Ashby and Cr^{51} methods was first stored in ACD solution in the blood bank refrigerator for twenty-four to thirty-six hours before its transfusion into the recipient.

The curve of survival of the autotransfused red blood cells in the 5 individuals is shown in Fig. 3. The two groups of curves developed by the radiochromium technique are similar as shown by a comparison of the curves in Figs. 2 and 3. Again the range of variation about a mean curve in the second radiochromium group is less than the variation about a mean of the 5 Ashby curves. Since there is no consistent difference in the two groups of curves

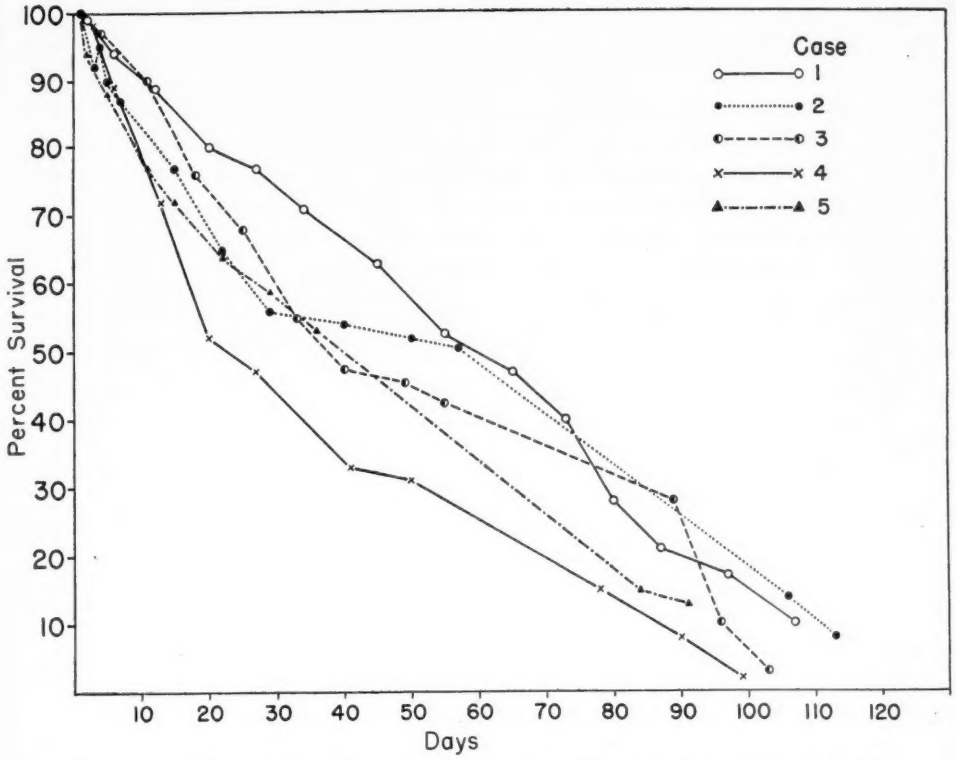

Fig. 1.—Erythrocyte survival of transfused heterotype blood by Ashby technique.

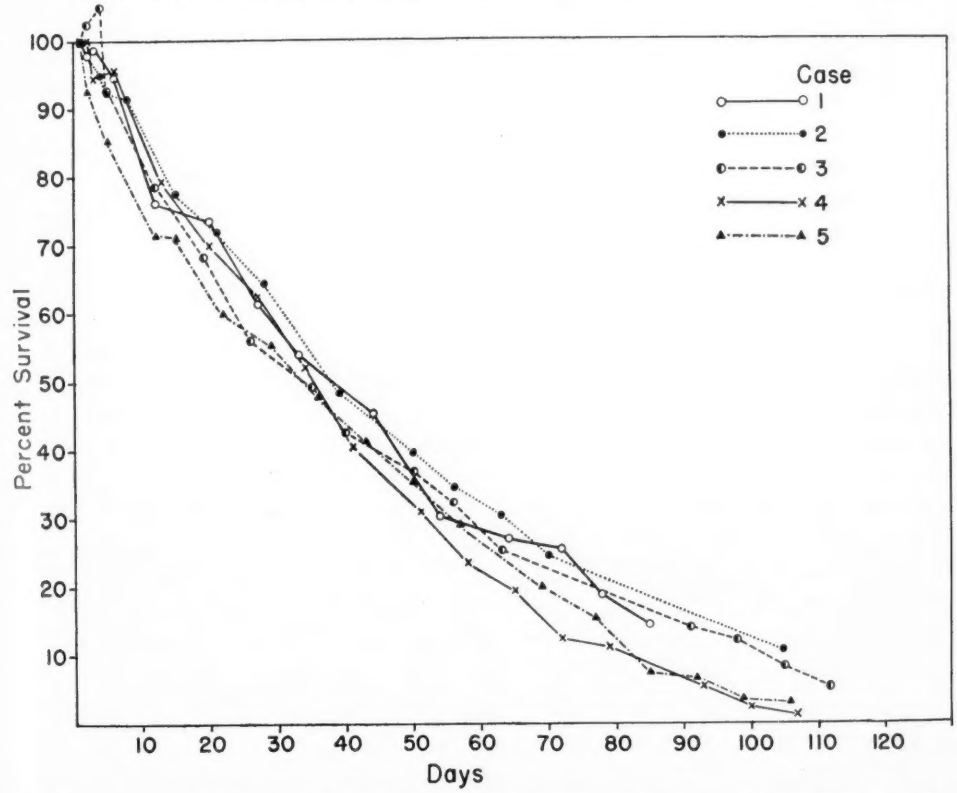

Fig. 2.—Erythrocyte survival of transfused heterotype blood by radioactive chromium technique.

developed by the radiochromium method, it appears that within the sensitivity and reliability of this method there is no difference in the survival of autotransfused blood as compared to the transfusion of compatible blood obtained from another individual (homotransfusion). The consistent curvilinear nature of the radiochromium curves as contrasted to the linear form of the classical Ashby red cell survival curve will be considered below.

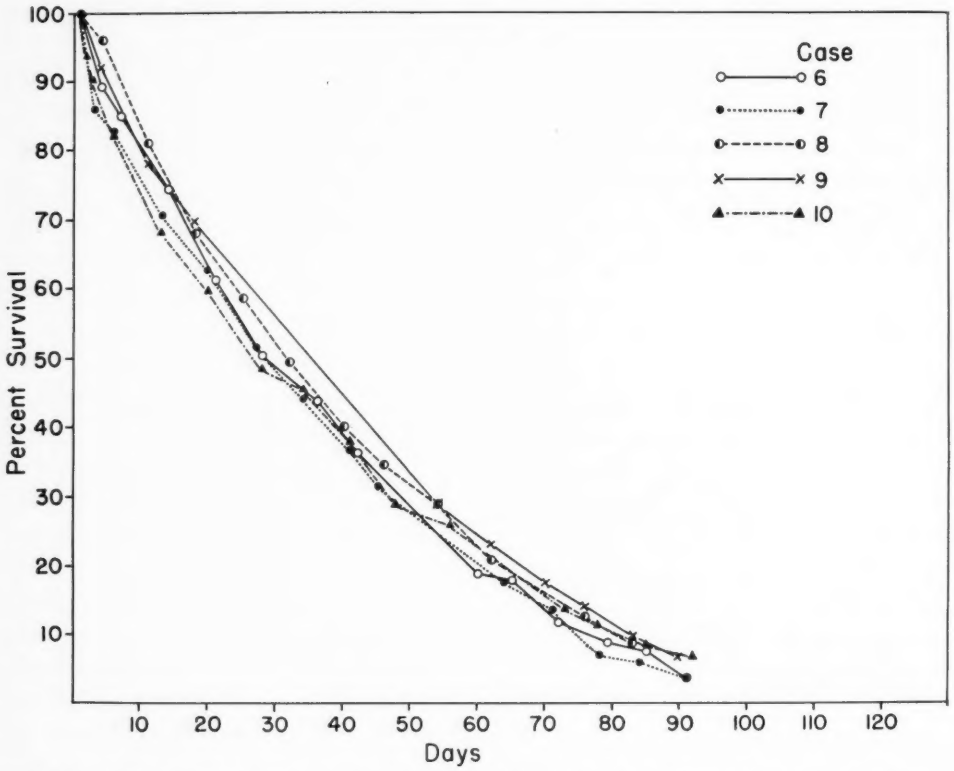

Fig. 3.—Erythrocyte survival of homotransfused blood by radioactive chromium technique.

A common expression of red cell survival is that of the "average percentage loss of cells per day." The normal range as determined by the Ashby technique is considered to be 0.85 to 1.1 per cent per day. A comparison of the two methods using this manner of expression is seen in Table II.

TABLE II. A COMPARISON OF ERYTHOCYTE DECAY RATES AS MEASURED SIMULTANEOUSLY BY THE ASHBY AND Cr^{51} METHODS

	ASHBY METHOD		Cr^{51} METHOD	
CASE NO.	PER CENT PER DAY LOSS	LENGTH OF STUDY IN DAYS	PER CENT PER DAY LOSS	LENGTH OF STUDY IN DAYS
1	0.88	97	1.0	85
2	0.82	113	0.85	105
3	0.91	103	0.85	112
4	0.98	99	0.94	107
5	0.95	91	0.91	106
Average	0.91		0.91	

The percentage of cells lost per day was determined at ninety days in both methods and in each of the five cases.

Preliminary observations indicate that the radiochromium technique, as herein described, demonstrates an increased rate of destruction of red blood cells in clinical hemolytic states. Examples of curves obtained in 4 such instances are seen in Fig. 4. These curves are compared graphically with the normal group. The hemolytic anemias resulted from uremia, pernicious anemia in relapse, and sickle-cell anemia.

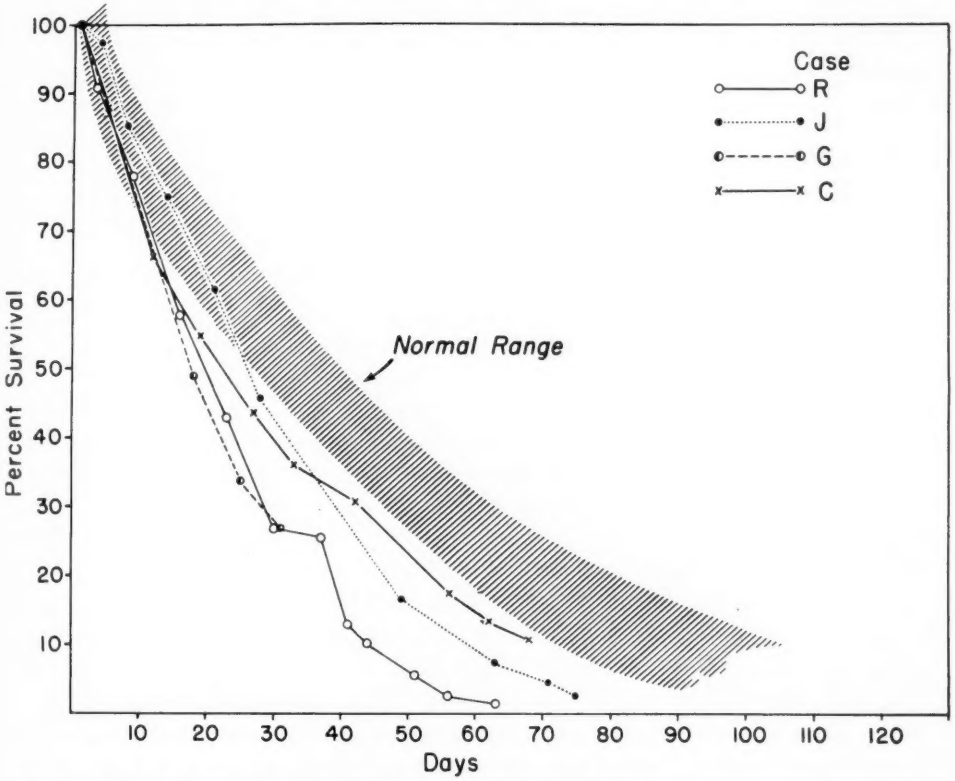

Fig. 4.—Erythrocyte survival in hemolytic anemias compared to range of normal group by radioactive chromium technique.

At the present time, studies are in progress which will attempt to determine the degree of variation between the radiochromium technique and the Ashby technique as applied to abnormal states and especially the various types of hemolytic anemia. Such a comparison appears necessary in order to determine whether or not the radiochromium per se or the tagging procedure alters the survival of the chromium tagged abnormal erythrocytes as judged by the survival characteristics of the untreated abnormal erythrocytes when determined by the Ashby method. Without a specific comparison between Cr^{51} treated and untreated erythrocytes there can be no quantitative comparison of the rate of abnormal hemolysis when one group is studied by the Ashby method and an entirely different group is studied by the radiochromium technique.

DISCUSSION

The method of determining the survival of normal red blood cells by means of the recession of radioactivity after tagging with radioactive chromium has yielded values similar to those observed by the simultaneous differential agglutination technique of Ashby. Moreover, the technique of measuring the relative number of surviving radiochromium tagged cells is precise and not subject to variations inherent in serologic tests. The validity of the radiochromium technique is strengthened by its application to the survival of rabbit erythrocytes.[16] The use of autogenous red cells tagged with radiochromium in the rabbit has yielded an exponential type curve of red cell degradation and ultimate survival characteristics (about sixty days) similar to those reported by Neuberger.[17] The correlations between the Ashby and the radiochromium methods in the normal human being, and the radiochromium method and the expected survival of rabbit erythrocytes as detected by N^{15} glycine, support the view that there is no significant in vivo leak of chromium from intact red cells.

Chromium has been reported to "leak" from intact erythrocytes at approximately 1 per cent per day.[14, 15] This constant was derived from in vitro studies. The possibility that such a loss might not occur during in vivo survival was suggested by the authors.[15] The in vitro phenomenon, however, was used by these workers to support an observation of "diminished survival" of normal human erythrocytes as obtained with the radiochromium method. The "apparent half survival time" of the normal erythrocytes was reported as 25 to 40 days.[15] We believe that this deviation from the expected values resulted not from an in vivo leak of the radiochromium but instead from the method used in expressing the data. This view is supported by the fact that our data, when expressed in the same manner as depicted by Necheles and co-workers[15] also yields an apparently shortened survival of red cells tagged with Cr^{51}. For instance, the erythrocytic survival in Cases 1 and 5 in our group is normal by both the Ashby and radiochromium techniques as shown in Figs. 1 and 2. The raw data that produced curves 1 and 5 in Fig. 2 can be re-treated to give the same abbreviated "apparent half survival" as illustrated in Fig. 5. The standard sample used was a diluted aliquot of the injected cells ordinarily utilized for determining the blood volume. Expressed in this way the range of the "apparent half survival" in the 10 cases in our study varied between 27 and 38 days with a mean value of 32.5 days.

It is suggested that data expressed in the latter manner, when converted to a means of expression similar to the Ashby method, yield survival characteristics similar to the accepted normal values. Our data do not support the hypothesis of an in vivo leak of radiochromium from intact red blood cells.

The curves in Figs. 2 and 3 are curvilinear and not linear. A replot of the data on semilogarithmic paper does not produce a straight line. The basis for this variation from the linear decay curve is unknown and its significance can be considered only in a speculative manner. Discussion of the theoretical aspects of factors involved in the normal and abnormal red cell decay curve

as obtained by the Ashby method has been presented by Sheets and associates,[18] Dornhorst,[19] and Evans and associates.[20] According to these discussions one hypothesis for the slightly curvilinear characteristic of the curves in the present study would include the possibility that a relatively mild random cell destruction was participating as well as cell death due to determinant cell life. Possibly the tagging procedure with radiochromium might alter the cells in such manner as to evoke this mild random mechanism. The fact that the curves approximate the pure linear function rather than the pure exponential function indicates that the random mechanism, if present, is relatively minor. It is of interest that a recent analysis of the red cell decay curve[20] has suggested a normal curvilinear function such as depicted in the radiochromium method herein described.

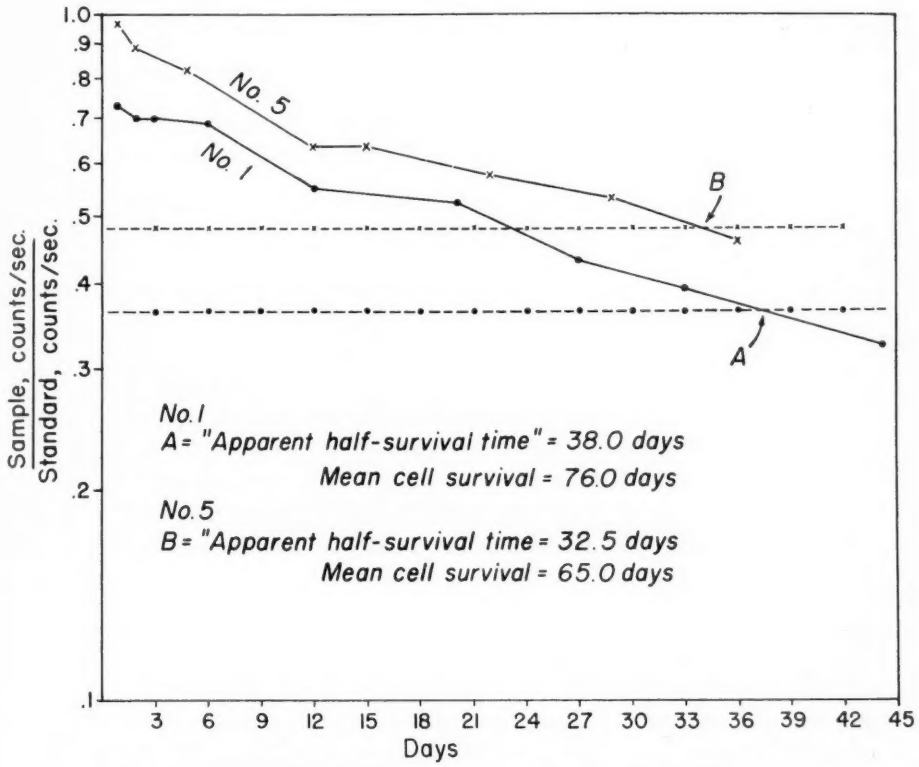

Fig. 5.—"Apparent half-survival time." (A replot of data from two cases.)

CONCLUSIONS

The radioactivity of normal human erythrocytes tagged in vitro with radioactive chromium describes a curve of recession within the circulation of normal individuals, which is comparable to the disappearance curve of non-agglutinable erythrocytes utilized in the Ashby differential agglutination method for the estimation of the survival of erythrocytes when both methods are applied at the same time. In order for the disappearance curve of Cr^{51}

tagged erythrocytes to resemble the Ashby curve the data derived from the former must be treated in a manner comparable to the treatment of the Ashby data.

Similar results have been obtained when the radioactive chromium tagging has been used with compatible heterotype erythrocytes used in the Ashby method (as type O given to a type A individual) and autogenous erythrocytes. The latter approach allows the estimation of the survival characteristics of erythrocytes within their own hemopoietic and circulatory systems.

The decay curve of radioactivity using heterotype or autogenous blood is slightly curvilinear. This feature has been discussed.

Preliminary observations suggest the feasibility of the use of the radiochromium technique herein described in the appraisal of hemolytic states.

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ENZYME ACTIVITY AND PROTEIN CONCENTRATION IN THE SERUM OF PATIENTS WITH MALNUTRITION

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THE activity of amylase and lipase was found to undergo characteristic and significant changes in starvation.[1, 2] The activity of these enzymes is also altered in the nutritional disorder of children known as kwashiorkor.[3, 4]

In the present paper a report is given on the changes of amylase and lipase activity in relation to the concentration of total proteins and of albumin, as found in cases of nutritional edema.

METHODS

a. The estimation of total proteins was performed in serum of patients (fasting for twelve hours) using either the micro-Kjeldahl technique or the method of Greenberg.[5]

b. Amylase was estimated according to the method of Smith and Roe.[6] The results are given in Smith-Roe units. (The activity of α-amylase is determined.)

c. Lipase was estimated with the method of Willstaetter and co-workers.[7] The results are expressed in Willstaetter units.

The data were examined statistically for correlation between enzyme activities and total protein and albumin concentration of serum; use was made of the analysis of variance to demonstrate the presence or absence of a statistically significant linearity of regression in between these variables.

The report is based on 28 pairs of estimations for serum amylase and lipase (in 15 patients); the enzyme activity in serum was estimated repeatedly together with the serum protein level in 4 patients as they recovered during treatment with high protein diet and protein concentrates† and finally reached normal protein levels in the serum. In the remaining cases, the values were obtained shortly after admission to the hospital Only such patients were included who did not suffer from any specific diseases, but showed characteristic signs of malnutrition; circulating plasma volume and thiocyanate space were estimated in all cases.[8]

RESULTS

Amylase.—The amylase activity of the serum varied over a range of 33 to 261 units in our cases, whereas the values for the albumin concentration showed a variation in between 1.1 and 4.5 Gm. per cent, being lowest for the low enzyme activity. Fig. 1 shows graphically the relation between the enzyme activity and the albumin concentration; the correlation coefficient ($\gamma = 0.71$) is statistically significant ($P = 0.001$); however, linearity of regression could be secured only up to an albumin concentration of 2.5 Gm. per cent. We conclude that a linear regression between albumin level and amylase activity of serum exists only within a limited range of the albumin concentration above which the enzyme activity is independent of the albumin concentration. In fact, the mean value of the amylase activity above 2.5 mg. per cent

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†Procasenol (Sharp & Dohme, Inc.)

albumin in serum is practically identical with the corresponding value of normal controls (200 ± 30.9). Linearity of the regression of albumin concentration upon amylase activity was not obtained for the full range of data, however, even if one of the variables was plotted on a logarithmic scale.

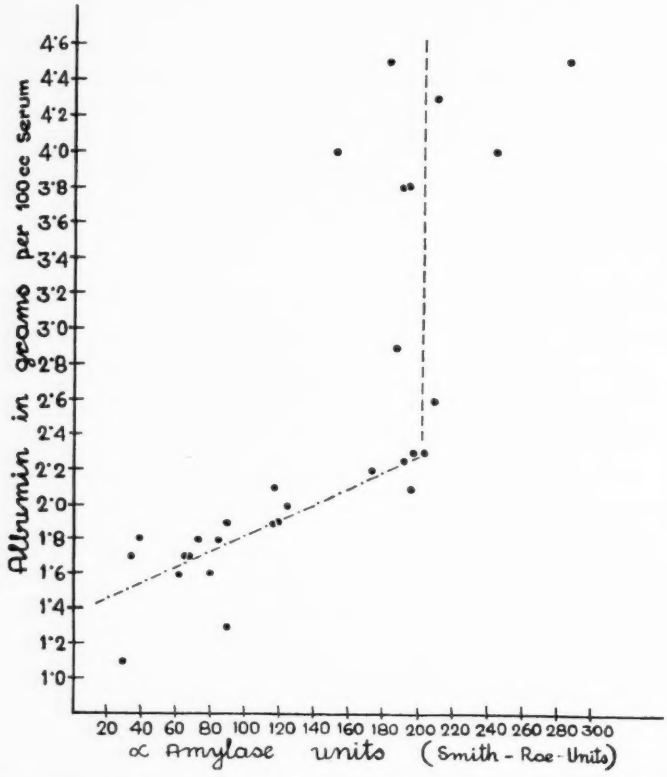

Fig. 1.—Graphical representation of α – amylase units (Smith-Roe) in relation to serum-albumin level (gram per cent) in patients of nutritional edema (including the values found after recovery).

—.—.—.—. Calculated regression line (linearity is statistically significant up to 2.5 Gm. per cent serum albumin).

---------- The mean value of the serum amylase activity found for serum albumin level higher than 2.5 Gm. per cent (= 200 ± 30.9); the value is identical with activities found in normal subjects.

It could be argued that the considerable change of circulating plasma volume observed in nutritional edema has some influence on these values. It was recently shown that the plasma volume is markedly reduced in the initial stages of nutritional edema, whereas an increase above normal takes place during the recovery phase.[8] Since, however, both albumin concentration and enzyme activity of the serum are affected in the same way by such volume changes of the circulating plasma, no serious error should be expected.

If the total protein concentration of serum, covering in our cases a range from 2.9 to 6.8 Gm. per cent, is taken as basis of reference, a correlation coefficient, $\gamma = 0.39$, is found which does not justify the assumption of a statistically significant correlation ($P > 0.05$).

Lipase.—Our results do not show a statistically significant correlation between the activity of lipase of serum (estimated as olive oil hydrolyzing activity) and the albumin or total protein concentration of serum over a range from 1.1 to 4.0 Gm. per cent albumin and 3.1 to 6.8 Gm. per cent total protein (15 patients); the enzyme activity varies around a similar mean value as found for normal subjects. In one case, however, the lipase activity of serum was on repeated estimation considerably lower; the enzyme activity (expressed in Willstaetter units) was 0.19, 0.19, and 0.28, respectively, in three estimations done at the interval of a fortnight each, and the corresponding albumin levels were 3.8, 3.6, and 3.1 Gm. per cent. The mean value of the serum lipase activity of the remaining 10 patients of that series was 1.01 ± 0.28; identical values were found for the serum lipase activity of 7 normal controls.

DISCUSSION

The cases of nutritional edema investigated here did not show a reduction of their lipase activity in serum, which is in contrast to the findings of Srinivasan and Patwardhan[4] and Trowell and co-workers[3] in a corresponding nutritional deficiency of children (kwashiorkor). Several points of interest arise out of this discrepancy: Kwashiorkor in children up to 5 years of age is invariably accompanied by more or less severe fatty infiltration of the liver[9] and by fibrosis of the pancreas.[3] Biopsy studies on the liver of patients suffering from nutritional edema did not reveal characteristic changes which can be found constantly; a certain degree of fatty infiltration may be present, but is not a regular finding[10]; some fibrosis might be present instead, or the liver showed an entirely normal appearance. It follows that the two conditions, nutritional edema and kwashiorkor in young children, cannot be identified with each other on morphologic grounds, and a similar conclusion should be drawn from the biochemical differences under discussion.

It is interesting to note that the serum amylase activity decreases only if the albumin concentration is below 2.5 Gm. per cent and shows a linear regression upon the albumin level only within a limited range below 2.5 Gm. per cent.

The influence of protein intake on enzyme activities was recently investigated for several enzymes: Ely and Ross[11] found by cytochemical and chemical means a reduction of acid phosphatase in the liver of rats, kept on a protein-free diet; alkaline phosphatase, however, showed increased activity under the same condition. The activity of pseudo-cholinesterase of rat liver decreases following short periods of fasting. It does not change, however, if reduced to nitrogen content of the tissue; obviously some endocrine influences regulate the activity of this enzyme in addition.[12] The catalase of liver was found to be very sensitive to starvation[13] and a similar condition was described for hepatic arginase.[14]

It must be stressed, however, that low protein intake in the diet is not systematically accompanied by low protein levels,[15] so that such results of feeding experiments cannot directly be compared with our results in which the enzyme activity was correlated with the protein level of serum. In neither

of these feeding experiments was a "threshold phenomenon" described similar to the one found here for amylase. It is conceivable that the threshold value of the serum albumin concentration, below which the enzyme activity of amylase decreases linearly, is only an expression for a deficiency of certain essential amino acids in tissue, which are indispensable for the synthesis of this particular protein. Such cases are known from the bacterial biosynthesis of certain enzymes.[16] In addition, the fact that various enzymes depend to a different extent on the total nitrogen content of the cells (for which the serum albumin level can be considered as a measure) has an analogy in bacterial metabolism.[17]

It was found in the present investigation during repeated estimations of the amylase activity in serum of the same patient that administration of a plentiful diet as such did not cause an increase of the lowered enzyme activity; only when the protein level started to rise during the treatment, was there a concomitant return of the enzyme activity to normal. In the case of cholinesterase of human serum, a correlation was found between the activity of the enzyme and the concentration of albumin in serum[18, 19] in various pathologic disorders. Based on their extensive studies of undernutrition, Hutchinson, McCance, and Widdowson[20] arrived, however, at the conclusion that a direct dependence of the enzyme activity on the dietary intake exists; an increase of the enzyme activity was found to take place on return to a plentiful diet, following a period of starvation, without a concomitant alteration of the serum protein concentration.

As pointed out before, no relation became obvious in between the food intake and the activity of serum amylase in our cases; it appears that the specific substrate of this enzyme (as offered in a plentiful diet) does not bear any influence on its activity. Considerations of the "mass action theory" of enzyme adaptation,[21] therefore, do not seem applicable in this case; it appears more compatible with the data to consider a shortage of pre-enzyme (precursor proteins) as the limiting factor for the formation of serum amylase.

SUMMARY

The activity of amylase and lipase was estimated in serum of patients suffering from nutritional edema.

A correlation was found (statistically significant at a P value of 0.001) between the amylase activity of serum and the serum albumin concentration: The albumin concentration varied over a range of 1.1 to 4.5 Gm. per cent, whereas the corresponding amylase activity varied between 33 and 261 units,[6] being lowest for the low albumin level. Return of the lowered amylase activity to normal was followed up in 4 patients during treatment and took place concomitantly with a rise of the serum albumin concentration. At albumin levels below 2.5 Gm. per cent, a linear regression exists between serum amylase and serum albumin concentration.

The activity of serum lipase was not significantly reduced as compared with normal control values (except in one out of 15 cases).

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THE EFFECT OF TESTOSTERONE PROPIONATE AT VARIOUS PROTEIN AND CALORIE INTAKES IN MALNUTRITION AFTER TRAUMA

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INTRODUCTION

AFTER severe trauma the combination of deficient intake of nutrients and the catabolic response to injury may lead to malnutrition. Nitrogen repletion is an important factor in recovery from malnutrition and in the healing of associated traumatic injuries.[1, 2] Testosterone has been used to minimize nitrogen loss or to aid in nitrogen repletion after trauma.[3-8] Administration of testosterone early in the posttraumatic state is beneficial.[4, 5, 7, 8] Use of the hormone, however, when the patient has spontaneously achieved positive nitrogen balance has been found to be without benefit.[6] Malnourished individuals without associated trauma store additional nitrogen when given testosterone,[9-11] but the results with the use of testosterone in starved rats have been contradictory.[12, 13]

In the majority of instances testosterone has been given to malnourished patients and the nitrogen and calorie intake kept constant. It is not clear what should be expected from the addition of testosterone, when the patient's intake of nitrogen or calories or both is increased, as usually occurs spontaneously in recovery from injury. Will testosterone have the same action with a high food intake as it did at a lower intake, or when the intake increases will testosterone add so little that its use is not worth while? The present study, in patients who became malnourished after severe injury, seeks to evaluate the use of testosterone at various adequate protein and calorie intakes.

MATERIALS AND METHODS

Four soldiers wounded in action in Korea were studied while under treatment with testosterone propionate.

Patient 1, a 23-year-old infantryman, height seventy-one inches, was in good health with an average weight of 82 kilograms until Oct. 14, 1952, when he sustained multiple penetrating wounds resulting in compound comminuted fractures of the right tibia and fibula and left femur, right hemopneumothorax, and multiple superficial lacerations of the head and neck. On Nov. 10, 1952, a right below-knee amputation was performed and on March 5, 1953, an intramedullary nail was placed in the fracture site of the left femur. During therapy the patient steadily lost weight. At the start of the present study on May 8, 1953, the patient weighed 45.80 kilograms. Physical examination revealed evidence of moderate weight loss, a well-healed right below-knee amputation stump, and a sinus tract at the site of femoral fracture draining purulent material from which was cultured hemolytic *Staphylococcus aureus* and *Escherichia coli*. Laboratory data for this and the following patients are shown in Table I.

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Patient 2, a 23-year-old tank commander, height sixty-seven inches, was in good health with an average weight of 66 kilograms until March 30, 1953, when a tank gun exploded resulting in second-degree burns of the hands, face, chest, abdomen, and thighs (approximately 20 per cent of the body surface); compound comminuted fracture of the right ilium; penetrating wound of the liver with disruption of the right hemidiaphragm and right hemopneumothorax; and avulsion of the skin and superficial muscles of the left upper abdomen and left lower chest. Following immediate operative repair of the liver laceration and disrupted right diaphragm, the patient received supportive treatment. At the start of the present study on May 19, 1953, the patient weighed 51.15 kilograms and appeared moderately underweight. All wounds had healed except an area of clean granulation tissue 30 by 15 cm. over the right upper thigh and right lower abdomen.

TABLE I. CALCULATIONS OF WEIGHT DISTRIBUTION BEFORE AND AFTER INJURY AND SELECTED BLOOD FINDINGS

	PATIENT			
	NO. 1	NO. 2	NO. 3	NO. 4
Before injury				
Preinjury weight (kg.)	82.0	66.0	61.5	80.0
Corrected preinjury weight (kg.*)	75.0	66.0	61.5	73.0
Preinjury lean body weight (kg.†)	68.8	61.7	60.2	66.6
Preinjury body fat (kg.)	6.2	4.3	1.3	6.4
On entry				
Weight on entry (kg.)	46.0	51.0	45.3	49.5
Lean body weight (kg.)	44.6	49.9	44.3	47.5
Body fat (kg.‡)	1.4	1.1	1.0	2.0
Per cent loss body weight	39	23	26	32
Per cent loss lean body weight	35	19	27	29
Per cent loss body fat	77	75	23	68
Red blood count (millions per cu. mm.)	4.5	4.5	4.7	4.8
Hemoglobin (Gm. per cent)	12.6	13.1	13.5	14.3
Hematocrit	44	45	46	47
Albumin (Gm. per cent)	3.54	3.79	4.11	4.23
Globulin (Gm. per cent)	3.45	3.72	3.89	3.03

*Corrected weight = body weight - weight of extremity lost.
†Calculated by formula of Behnke. (See text.)
‡Skinfold measurement. (See text.)

Patient 3, a 20-year-old infantryman, height sixty-eight inches, was in good health with an average weight of 61.5 kilograms until April 15, 1953, when he was wounded by small arms fire resulting in avulsion of the anterior two-thirds of the maxilla and hard palate, laceration of the tongue, and perforation of the sigmoid colon. Immediate repair of the facial defect was begun, and the perforated sigmoid colon was exteriorized until April 24, 1953. Liquid diet was begun on April 21, 1953, and increased as rapidly as possible, but the patient continued to lose weight slowly. At the start of the present study on June 4, 1953, the patient weighed 45.30 kilograms and appeared markedly underweight. The abdominal and facial wounds were well healed.

Patient 4, a 21-year-old infantryman, height seventy inches, was in good health with an average weight of 80 kilograms until Feb. 21, 1953, when he was wounded by small arms fire resulting in multiple perforations of the colon and small intestine and severance of the left femoral artery and vein. Laparotomy with repair of the intestinal perforations, and anastomosis of the severed left femoral artery were performed immediately. Amputation of the left leg below the knee was carried out on March 2, 1953, because of gangrene of the extremity. On March 11, 1953, a left above-knee amputation was performed because of gangrene of the previous amputation stump. At the start of the present study on July 7, 1953, the patient weighed 49.50 kilograms and appeared moderately underweight. All wounds were well healed.

Each patient except Patient 4 received a liquid diet. Methods utilized and the technique of nitrogen balance as carried out at this installation have been reported elsewhere.[14]

Body weight was determined daily, after voiding, before breakfast, on a scale accurate to ± 25 Gm. Preinjury lean body weight was calculated by the formula of Behnke,[15] where lean body weight = 53.9 (surface area in m^2) −32.6. Body fat on entry as shown in Table I

TABLE II. SUMMARY AND STATISTICAL ANALYSIS OF BALANCE DATA

PATIENT AND PERIOD	DATE	DAILY CALORIE INTAKE	DAILY NITROGEN INTAKE (GM.)	TREATMENT	MEAN NITROGEN RETAINED (MG. PER KG. PER DAY) ± S.E. MEAN	STATISTICAL ANALYSIS‡
No. 1						
1.	May 14-May 18	3,000	31.7	None	149 ± 5	
2.	May 23-May 25	3,000	31.7	TP 25 mg.	181 ± 3	Period 1 vs. Period 2 $p = <0.01$
3.	May 27-May 29	3,000	31.7	TP 50 mg.	203 ± 4	Period 2 vs. Period 3 $p = 0.02$
4.	June 13-June 15	3,000	15.5	None	49 ± 7	
5.	June 19-June 21	3,000	15.5	TP 25 mg.	86 ± 6	Period 4 vs. Period 5 $p = <0.05$
6.	June 23-June 26	3,000	15.5	TP 50 mg.	102 ± 5	Period 5 vs. Period 6 $p = <0.1$
No. 2						
1.	May 22-May 25	3,300	15.9	None	99 ± 3	
2.	June 1-June 3	3,300	15.7	TP 25 mg.	115 ± 8 (102*)	Period 1 vs. Period 2 $p = 0.1$
3.	June 5-June 8	3,300	15.7	TP 50 mg.	134 ± 7 (119*)	Period 1 vs. Period 3 $p = <0.01$: Period 2 vs. Period 3 $p = <0.05$
4.	June 12-June 14	2,600	15.8	None	57 ± 10	
5.	June 17-June 20	2,600	15.8	TP 25 mg.	83 ± 5	Period 4 vs. Period 5 $p = <0.05$
6.	June 22-June 24	2,600	15.8	TP 50 mg.	116 ± 3	Period 5 vs. Period 6 $p = <0.01$
7.	July 17-July 23	3,300	28.2	None	106 ± 5	
8.	July 27-July 31	3,300	28.2	TP 25 mg.	142 ± 7	Period 7 vs. Period 8 $p = <0.01$
No. 3						
1.	June 6-June 10	2,000	15.7	None	85 ± 5	
2.	June 16-June 18	2,000	15.7	TP 25 mg.	135	
3.	Sept. 12-Sept. 15	3,000	14.9	None	64 ± 3	
4.	Sept. 22-Sept. 24	3,000	18.1	TP 25 mg.	153	
No. 4						
1.	July 9-July 13	2,340	17.5	None	55 ± 4	
2.	July 16-July 19	2,340	17.5	TP 25 mg.	101 ± 4	Period 1 vs. Period 2 $p = <0.01$
3.	July 23-July 26	3,140	17.4	TP 25 mg.	135 ± 4 (119†)	

*Corrected to caloric intake of 2,600 cal. per day. (See text.)
†Corrected to caloric intake of 2,340 cal. per day.

‡ $$t = \frac{\bar{Y}_1 - \bar{Y}_2}{s}\sqrt{\frac{n_1 \times n_2}{n_1 + n_2}}$$

was determined by skinfold measurement as described by Brozek and Keys[16] with use of the formula of Rathbun and Pace[17] to convert body specific gravity to percentage of body fat.

RESULTS

Figs. 1 to 5 illustrate the results of the present studies in detail. Table II summarizes these results and presents the statistical analysis of the data.

In Fig. 1 the response of Patient 1 to testosterone is shown with diets containing 31.7 Gm. and 15.5 Gm. nitrogen per day and 3,000 calories per day. Testosterone added significant additional nitrogen retention during both diets. The difference in nitrogen retention produced by 25 and 50 mg. testosterone propionate is significant with the high nitrogen diet but equivocal with the low nitrogen diet. Electrolyte balance studies in this patient and in Patient 2 (Fig. 3) indicate that sodium retention was not a major problem, and that potassium balance study gave little additional information. No additional electrolyte balances were taken.

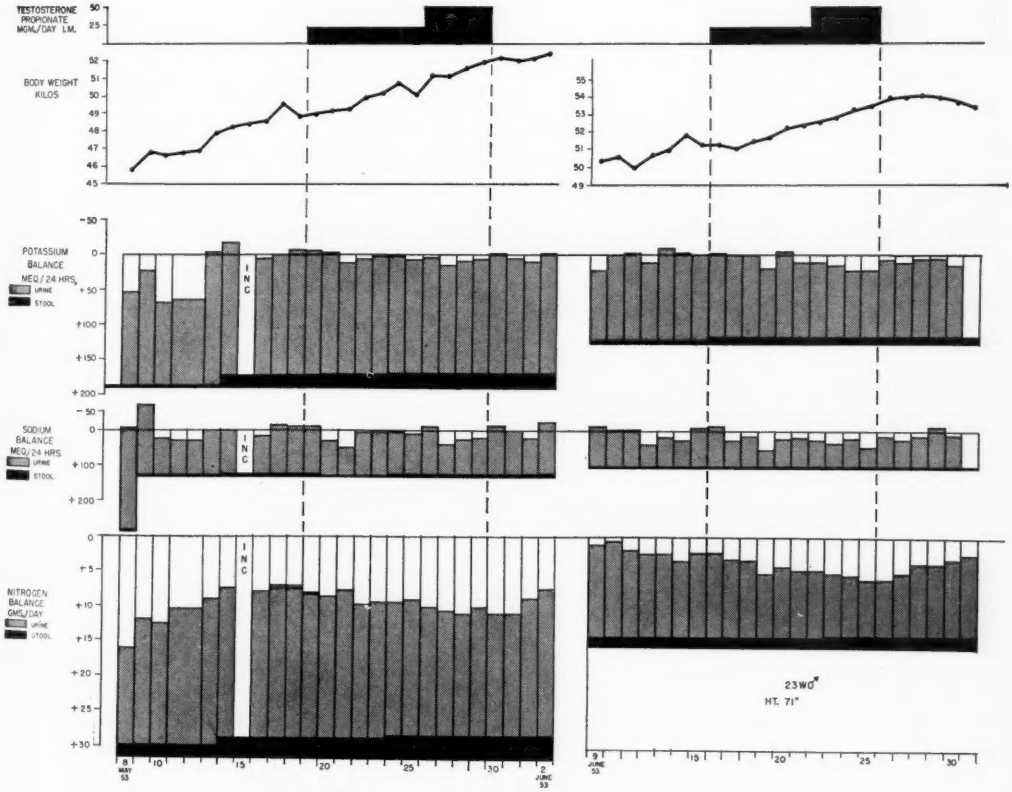

Fig. 1.—Balance data of Patient 1. The diet is isocaloric at the two levels of nitrogen intake. *Inc* indicates a day of incomplete urine collection.

Fig. 2 illustrates the effect of testosterone on Patient 2 in diets containing 15.8 Gm. and 28.2 Gm. nitrogen per day and 3,300 calories per day. Testosterone adds significantly to nitrogen retention at both levels of nitrogen intake.

With a constant nitrogen intake of 15.8 Gm. per day the calorie content of the diet was varied in Patient 2 as shown in Fig. 3. The use of testosterone led to additional nitrogen retention with both diets.

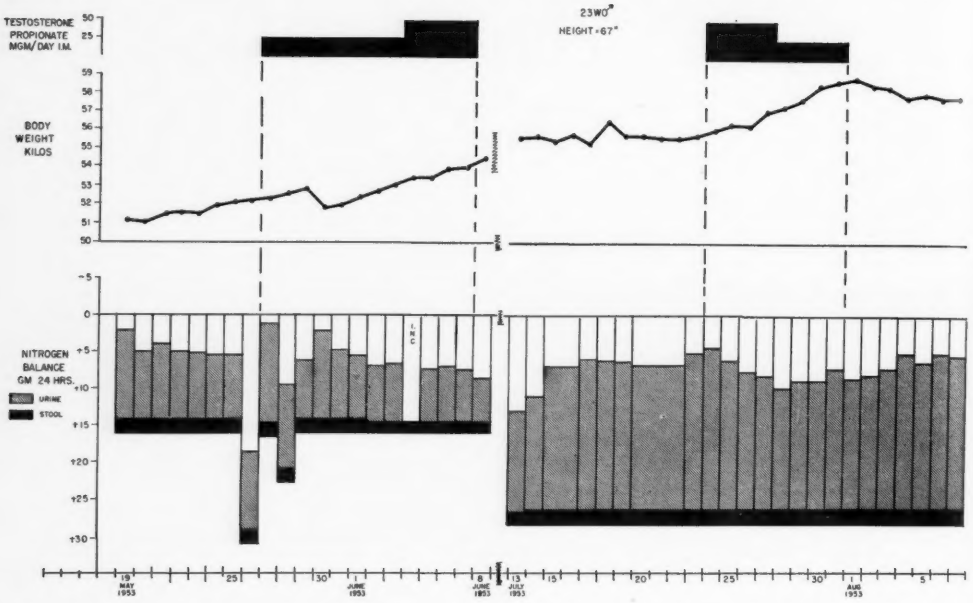

Fig. 2.—Nitrogen balance data of Patient 2 with two levels of nitrogen intake. The diets are isocaloric.

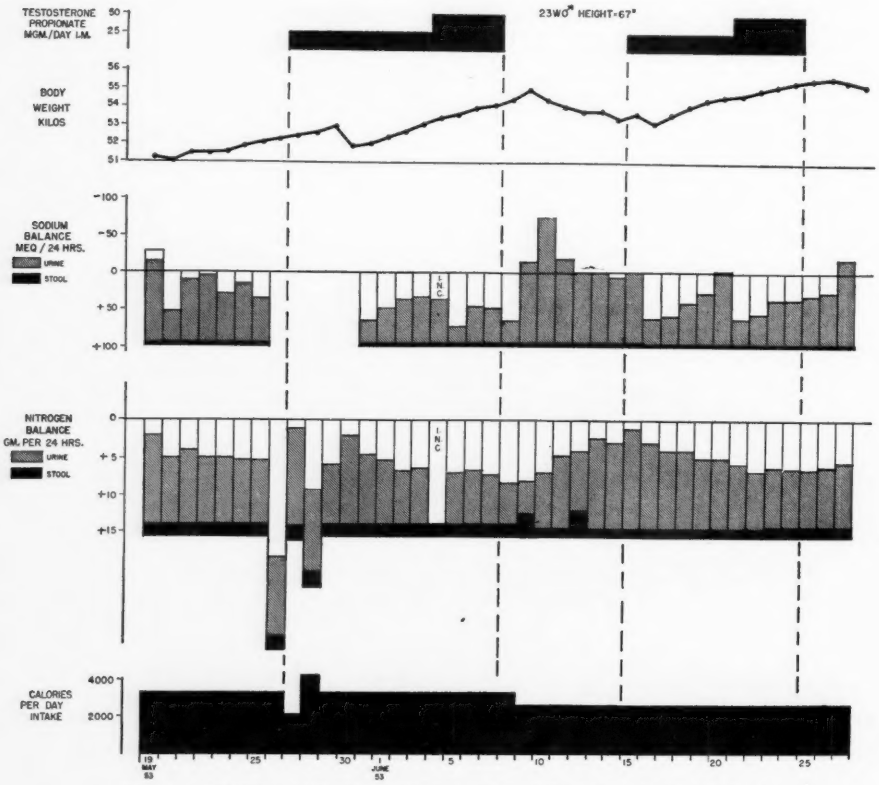

Fig. 3.—Balance data of Patient 2 with different caloric intakes.

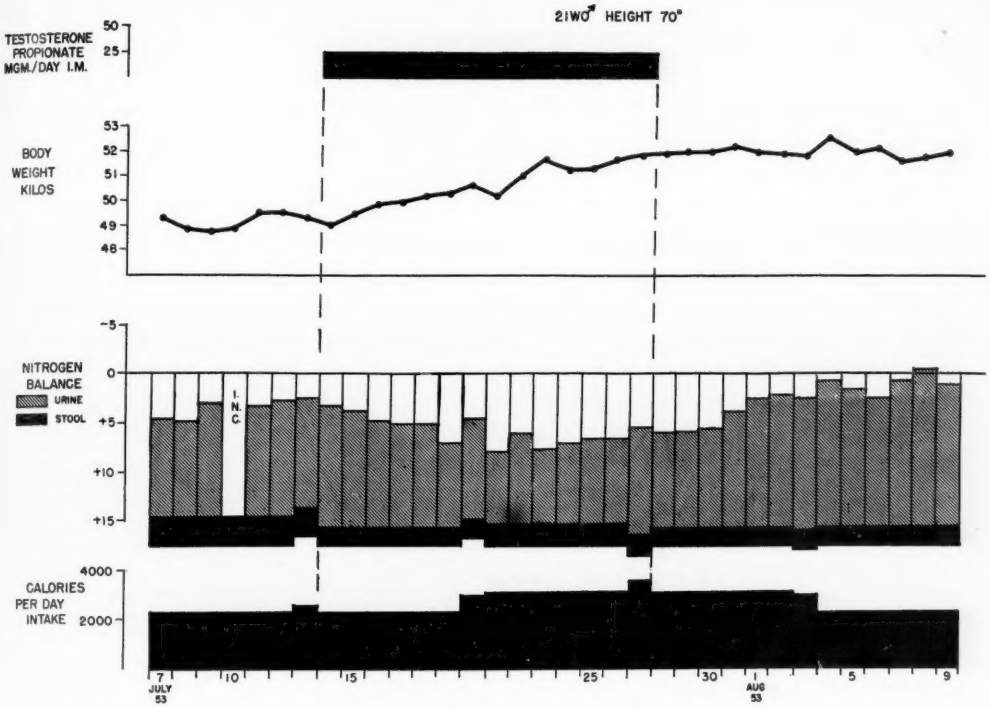

Fig. 4.—Balance data of Patient 4 with variation of caloric intake.

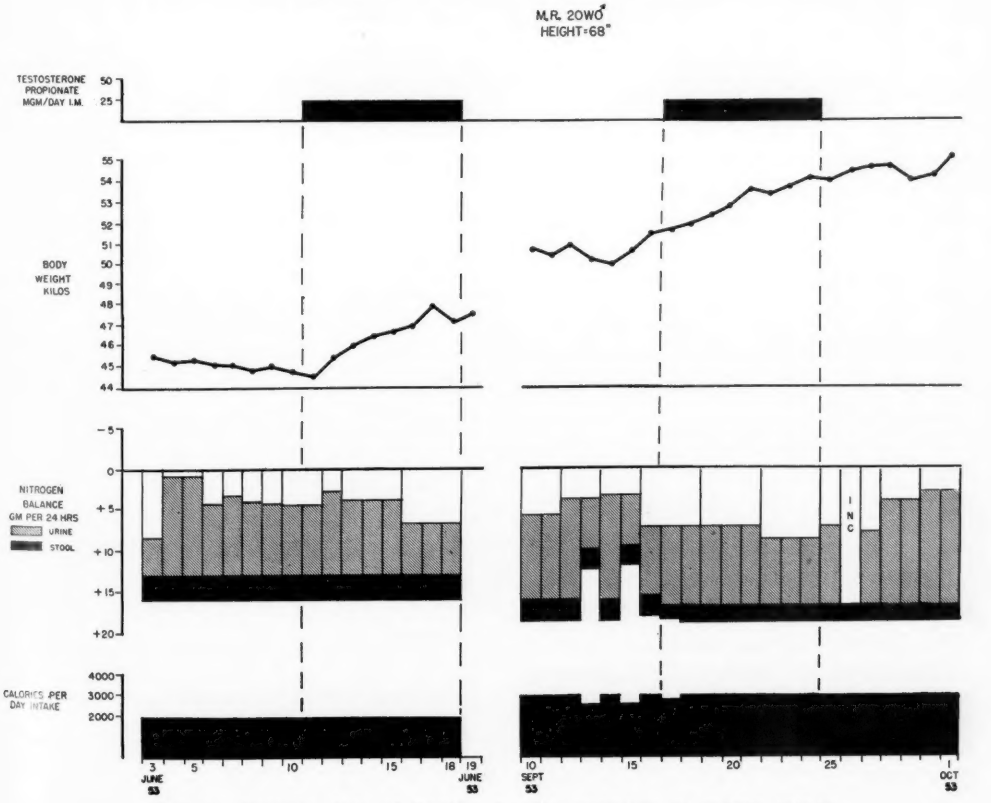

Fig. 5.—Balance data of Patient 3 with variation of caloric intake.

As illustrated in Fig. 4, the caloric intake of Patient 4 was increased while the patient was receiving testosterone and a constant nitrogen intake without altering significantly the effect of testosterone.

It has been shown that each calorie added per day to a maintenance diet leads to retention of approximately 1 mg. of nitrogen per day.[18, 19] Utilizing this value it becomes possible to subtract the effect of the calories per se at higher caloric levels and so observe the effect of testosterone alone on nitrogen retention. Introduction of such calculation is shown in Table II and leads to the conclusion that in the studies represented in Figs. 3 and 4, testosterone has the same effect at different levels of caloric intake.

Because of language difficulties with Patient 3 it was necessary to pool several days' urines on a number of occasions. It became impossible to compare so few analytic values statistically. Fig. 5 depicts the additional nitrogen retained when testosterone was administered.

DISCUSSION

In the malnourished patients studied, nitrogen retention was increased significantly over control amounts by the administration of testosterone propionate (TP) when diets containing between 15.5 and 31.7 Gm. N per day were fed. Within these limits, however, the actual protein content of the diet did not consistently influence the degree of nitrogen retention induced by TP. For example, Patient 1, while receiving 25 mg. TP daily, retained an extra 33 mg. N per kilogram per day on a dietary intake of 31.7 Gm. N per day and an extra 37 mg. N per kilogram per day on a diet of 15.5 Gm. N per day. When given 50 mg. TP daily, he retained an extra 54 mg. N per kilogram per day on the former diet and an extra 53 mg. N per kilogram per day on the latter diet. This indicates that the factor controlling the amount of added nitrogen retention was the TP and not the protein intake. Patient 2, on the other hand, when given 25 mg. TP daily retained 16 mg. N per kilogram per day on an intake of 15.9 Gm. nitrogen and 36 mg. per kilogram per day on an intake of 28.2 Gm. N per day. Although the dietary level of nitrogen appears here to influence the degree of nitrogen retention, these differences are not statistically significant ($p = >0.05$). Furthermore, the levels of nitrogen retained on the same dosage of TP were not constant in different individuals. Patient 4 retained an extra 46 mg. N per kilogram per day when given 25 mg. TP and a diet containing 17.5 Gm. N per day, somewhat greater retention than in the other two patients.

These findings are similar to those reported by Butler and associates[20] who, in a study of a fasting man, found that 25 mg. TP daily induced nitrogen retention of 33 mg. per kilogram per day. Studies in normally nourished rats have also shown no alteration of testosterone effect when dietary nitrogen was varied.[21] Taken together, these data suggest that dietary nitrogen intake has no effect on the magnitude of nitrogen retention induced by testosterone.

Similarly, caloric intake appears to have little effect on the magnitude of nitrogen retention with TP if the intake is adequate (Table II). Patient 2, for example, when given TP 25 mg. per day retained 82 mg. per kilogram per

day on the 2,600 calorie diet and 102 mg. N per kilogram per day on the 3,300 calorie diet corrected to 2,600 calories, while with 50 mg. TP daily, 116 mg. N per kilogram per day and 119 mg. N per kilogram per day were retained on the respective diets. The effect of testosterone at different inadequate caloric intakes may, however, vary. In the study of Butler and associates,[20] TP 25 mg. per day led to greater retention of nitrogen in a fasting subject than in the subject who received 300 Gm. glucose per day.

The patients in the present study were begun on testosterone from fifty-seven to 207 days after their initial severe injuries and all were in positive nitrogen balance at the time testosterone was begun. Nevertheless, they all showed significant additional nitrogen retention with testosterone. This is to be contrasted with the patients of Cooper and associates,[6] who showed no added nitrogen retention with TP if they were already in positive nitrogen balance. The two groups of patients are, however, greatly different in the degree of weight loss each sustained. Since patients comparable to those in the present series apparently have an unlimited ability to retain nitrogen as the dietary nitrogen is increased,[22, 23] it is not surprising that use of testosterone leads to additional nitrogen retention.

After injury, Patients 1, 4, 3, and 2 lost, respectively, 35, 29, 27, and 19 per cent of their lean body weight. No consistent relationship is apparent in this small series between the amount of lean body weight lost and the additional nitrogen retained with a given dose of TP. It would appear from these limited data that the degree of response to TP is not clearly related to degree of weight loss.

SUMMARY

Testosterone propionate is useful in the treatment of malnutrition following severe trauma. Additional nitrogen retention can be produced even when the patient is in positive nitrogen balance. The degree of response to testosterone is not related to the amount of lean body weight lost. In the individual patient the additional nitrogen retention produced by testosterone was the same at various adequate nitrogen and caloric intakes.

We wish to thank Captain Johnnie Long and the nursing staff of the Metabolic Ward, First Lieutenant Margaret E. Shipman and the dietetic staff, and Betty Hackley, Norman Trachtenberg, and Marjorie Knowlton for their invaluable assistance with these studies.

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THE VALUE OF SERUM ALKALINE PHOSPHATASE DETERMINATION AND BROMSULPHALEIN TEST IN THE DIAGNOSIS OF METASTATIC CANCER OF THE LIVER

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IN SPITE of hopeless prospects at the present time for the patient with malignant metastasis of the liver, a method for making an early diagnosis of such metastasis should be of more than academic interest, especially when the metastasis occurs some time after the resection of the primary growth. To be able to suspect liver metastasis with fair assurance should be useful to the physician to help him avoid groping for an explanation for the vague complaints of the patient that may be present during the earlier stages of this complication, as well as to enable him to warn immediate members of the family of the impending disaster.

The frequency with which liver metastasis occurs from cancer in any part of the body and especially from those areas drained by the portal vein makes early recognition of such metastasis highly desirable. Satterlee[1] has estimated that according to the laws of probability, 36 per cent of all cancers will metastasize to the liver and 50 per cent of portal cancers will invade this organ.

Although Flood, Gutman, and Gutman,[2] in 1937, reported increased serum alkaline phosphatase concentration in seventeen of twenty cases of neoplasm with proved involvement of the liver, and Meranze and his associates[3] again stressed the possible significance of an elevation of this enzyme in the serum to liver metastasis, little attention appears to have been paid to their findings from a review of the literature of the past fifteen years. In a recent review of liver function tests, Mateer and associates[4] suggested only the Bromsulphalein and hippuric acid tests in the diagnosis of cancer of the liver, and as useful in a study specifically devoted to this problem, Thomas and Zimmerman[5] employed the Bromsulphalein, thymol turbidity, and cephalin-cholesterol flocculation tests and made no mention of serum alkaline phosphatase. Only recently have Bullard[6] and Cabello, Silva, and Icekson[7] reported on the diagnostic value of serum alkaline phosphatase determination in hepatic metastases. Our own studies in the past few years indicate that the elevation of serum alkaline phosphatase in the absence of hyperbilirubinemia, in a patient who is to have or has had a resection of a primary cancer, constitutes the closest that any liver function test approaches some degree of specificity for arousing suspicion of liver metastases. This specificity, as we shall see, may be further enhanced by combining it with the Bromsulphalein test.

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PROCEDURES

Serum bilirubin was measured by the quantitative van den Bergh reaction using the method of Malloy and Evelyn.[8] Serum phosphatase was determined by the Shinowara, Reinhart, Jones[9] technique with which the upper limit of normal is 9 units. The degree of Bromsulphalein retention was determined electrophotocolorimetrically thirty minutes after the intravenous injection of 2 mg. per kilogram body weight of the dye. By this method, up to 4 per cent of the dye may be found in the blood serum at thirty minutes. During the past several years, we have returned to the use of the 2 mg. instead of the 5 mg. per kilogram dose of dye, because Lorber and Shay[10] have demonstrated an enterohepatic circulation of this dye and have shown that this factor may add appreciable increments of dye to the blood serum level after the 5 mg. per kilogram dose.

DATA AND DISCUSSION

Table I lists eighteen patients with primary carcinoma of some portion of the gastrointestinal wall and metastasis to the liver, confirmed at operation, in whom preoperative total serum bilirubin levels were all below 1 mg. per cent, except one (1.07 mg.), and all of whom showed serum alkaline phosphatase levels above 9 units. Nine, in whom the Bromsulphalein test was also made, showed abnormal levels of serum retention of the dye.

TABLE I. PROVED CASES OF METASTATIC CARCINOMA OF THE LIVER

CASE	SERUM BILIRUBIN		ALKALINE P'TASE	B.S.P. 2 MG./KG. DOSE
	I MIN.	TOTAL		
H. B.	0.38	0.83	16.9	—
W. S. J.	0.25	0.73	23.6	25.5
H. K.	0.03	0.39	12.2	—
R. M.	0.0	0.67	14.4	—
E. S.	0.19	0.70	19.5	—
K. W.	0.31	0.95	10.4	20.7
E. So.	0.19	0.52	37.2	18.5
G. M.	0.15	0.79	11.5	11.8
D. P.	0.12	0.49	10.1	16.6
H. W.	0.42	1.07	26.6	—
B. G.	0.15	0.97	18.4	7.9
R. M.	0.18	0.91	28.3	18.8
I. G.	0.06	0.58	14.7	—
M. G.	0.05	0.51	12.8	—
M. B.	0.18	0.55	25.5	15.6
J. R.	0.13	0.53	11.9	5.2
J. P.	0.06	0.41	11.7	—
R. E.	0.27	0.83	14.9	—

This table can serve as the text for a consideration of one aspect of the problem, namely, what reliance can be placed on the results of these tests when the patient is seen for the first time? We advocate use of both serum phosphatase level and Bromsulphalein test because the result of one increases the specificity of the other. Let us emphasize that the use of the term "specificity" represents a certain poetic license since we do not mean to imply that either test alone or the two in combination will spell out liver malignancy. An abnormal Bromsulphalein level in the blood serum can only be interpreted as evidence of liver disease that is interfering with the excretory function of that organ, and an elevated serum alkaline phosphatase, if due to the liver, represents either an interference with the excretion of the phosphatase formed

by that organ, an overproduction of this enzyme by the liver, or a combination of both conditions. If the serum phosphatase level is considered without benefit of the results of the Bromsulphalein test, an elevation of this enzyme may well have its origin in some bone disease. While an elevated serum Bromsulphalein retention does not necessarily stamp an associated elevated serum alkaline phosphatase as of liver origin, it is then more likely to be of that origin. In this way, an abnormal, Bromsulphalein test serves to increase the specificity of an elevated serum alkaline phosphatase as due to disease in the liver. Conversely, an elevated serum phosphatase in the presence of a normal serum bilirubin makes an abnormal Bromsulphalein response more likely to be due to hepatic neoplasm than if such a result were an isolated finding.

Patients in whom a normal Bromsulphalein elimination or a normal serum phosphatase value was found have been reported; yet at operation, metastatic carcinoma of the liver was present. While we have not, as yet, encountered such "false negative" results, we probably will, since we are not aware of the minimal liver changes necessary to make either test positive. Cabello and his associates[7] failed to find an elevated serum phosphatase in 11 per cent of forty-two patients with proved hepatic metastasis, and Bullard[6] had a similar experience in six of his series.

All investigators who have studied either the serum alkaline phosphatase or the Bromsulphalein test in relation to this problem have encountered patients with abnormal values for either test in whom, at operation for the primary lesion, the surgeon was unable to confirm the diagnosis of hepatic metastasis. Such "false positive" results, however, should not be accepted as readily as an error as when the surgeon finds obvious metastasis with false negative values, for, at operation, the surgeon is limited in his examination of the liver only to those areas of the surface of the organ which he can see or feel and can say nothing of possible lesions within the body of the liver itself. Such false positive cases, if no other suitable explanation for the abnormal test is found, should be followed for at least one year before one can be certain that no liver involvement was really present.

Let us hasten to emphasize, however, that hepatic lesions other than primary or metastatic carcinoma of the liver may, at times, produce elevated serum alkaline phosphatase and increasd Bromsulphalein retention without an elevation of serum bilirubin. Many years ago, the senior author[11] first stressed the importance of serial liver function profile studies in any attempt to evaluate liver function. Experience over the years has not only justified this point of view in our own work, but has come to be generally accepted as the method of study regardless of which tests are selected for the profile, for the changing functional picture of the liver obtained in this way stands in the same relationship to any single study as does the moving picture to a single snapshot. Such repeated studies will avoid one of the pitfalls of a too rigid interpretation of any one laboratory study. Patient E. M. is a case in point and illustrates that truly false positive (for example, no organic liver involvement) results may occur. This patient, a 68-year-old woman, had given a history suggestive of biliary tract disease with attacks of biliary colic for several years which had

TABLE II. PATIENT E. M.: GALLSTONES—HYPERTENSION

DATE	SERUM BILIRUBIN		CEPH.	COLL.	THYMOL TURBIDITY		GAMMA	ALK.	SERUM LIPIDS					2 MG.
									CHOLESTEROL		PHOSPHO.	NEUT.	TOTAL	
	1 MIN.	TOTAL	CHOL.	GOLD	UNITS	18 HR. %	GLOBULIN	P'TASE	TOTAL	E/T	LIPIDS	FAT	LIPIDS	BROM.
NORMAL	< 0.25	< 1.00	< +2	< +1	< 4.0	> 85	< 8.0	< 9.0	200 ± 50	50 - 70	200 ± 50	< 350	720 ± 200	< 4 %
4- 5-51	0.43	0.63	-	-	1.2	108	4.7	36.5	228	47	183	48	531	16.5
4-12-51														5.0
4-19-51	0.24	0.40	-	-	1.2	115	7.5	20.8						5.9
4-26-51	0.06	0.43	-	-	1.5	100	7.0	19.1						2.9
5- 8-51	0.16	0.45						20.7						3.8
5-29-51	0	0.31	-	-	1.3	115	6.7	9.47						4.0
6-19-51	0	0.24	-	-	1.4	100	7.9	5.29						2.3
9-25-52	0.28	0.85	+1	-	1.2	108	5.1	26.3	229	36				12.2
10-16-52	0.48	0.61	±	-	1.0	80	4.9	40.21						14
10-17-52	*CHOLECYSTECTOMY*													
10-18-52								37.81						
10-20-52	0.20	0.63						23.77						
10-23-52	0.13	0.46						17.79						
10-25-52	0.09	0.33						13.59						
10-27-52								10.77						
10-30-52	0.13	0.21	+2	-	0.9	111	4.4	7.85						3.3
10-31-52								9.40						
11-11-52	0.13	0.27						6.27						1.7
2-19-53	0.12	0.45						4.07						2.4

been accompanied, at least in some of the attacks, by hyperbilirubinemia. Cholecystography had disclosed gallstones. The other pertinent feature of her history was a weight loss of 15 pounds in the preceding three or four months. The results of a liver function profile on April 5, 1951 (Table II), made it necessary to consider liver metastasis because of the high Bromsulphalein retention and marked elevation of serum alkaline phosphatase accompanying a normal serum bilirubin. Within two weeks Bromsulphalein retention had returned to normal and serum alkaline phosphatase showed a sharp drop. The patient refused operation and repeat liver function studies about ten weeks (June 19, 1951) after the initial studies were entirely normal. The liver function changes noted at the beginning of these studies had to be attributed to the biliary tract disease and not to malignant metastasis since we have never seen a significant return toward normal of either the Bromsulphalein retention or the serum alkaline phosphatase once they had reached the levels in this patient, if they were really due to liver neoplasm. She was not seen again for fifteen months, when she returned with a history of repeated attacks of unexplained chills and fever with elevations of temperature to 104° F., lasting from twelve to twenty-four hours, without any pain. Liver function profile gave the results in Table II, recorded under date of Sept. 25, 1952. Abdominal examination revealed no abnormalities. There was no tenderness under the right costal margin, the liver was not enlarged, and nothing was felt that suggested a palpable gall bladder. Her temperature was now normal. She was hospitalized on Oct. 10, 1952. On the third hospital day she developed a chill and her temperature rose to 102° F. She had no pain. Abdominal examination uncovered no area of tenderness but on the right side of the abdomen a nontender, large, sausage-shaped mass, a distended gall bladder, was found. On this day (Oct. 16, 1952) liver function profile again showed abnormal Bromsulphalein retention, marked elevation of serum alkaline phosphatase, and a normal serum bilirubin. The one-minute bilirubin was elevated. She was operated upon Oct. 17, 1952, when an uncomplicated cholecystitis with stones and a normal-looking liver were found. Subsequently, liver function tests all returned to normal and have remained so since. This is the only patient in our series of many with gallstone disease in whom such strikingly abnormal results in serum phosphatase and Bromsulphalein were found without any elevation in serum bilirubin.

It is important, too, in testing liver function in a patient in whom a metastatic liver carcinoma may be present, that the tests not be limited to the three already indicated, but that a reasonably complete liver function profile be obtained. For many years we have employed the following group of tests in studying liver functions: one-minute and total serum bilirubin, cephalin cholesterol, colloidal gold, thymol turbidity, eighteen-hour turbidity ratio and gamma globulin flocculation tests, serum alkaline phosphatase, total cholesterol and cholesterol esters when jaundice is present, and, in addition, the Bromsulphalein test when the patient is not jaundiced. These tests have been chosen for two reasons[12]: one, they include two groups of tests that are primarily modified by obstruction in the biliary tree (serum alkaline phosphatase and

TABLE III. LIVER METASTASIS. CARCINOMA OF COLON

♂ I. GI. AGE 67	SERUM BILIRUBIN		CEPH. CHOL.	COLL. GOLD	THYMOL TURBIDITY		γ GLOB.	ALK. P'TASE.	SERUM LIPIDS				
									CHOLESTEROL		PHOSPHO.	NEUTRAL	TOTAL
	1 MIN.	TOTAL			UNITS	18 HR. RATIO			TOTAL	E/T	LIPIDS	FAT	LIPIDS
Operation—November, 1947													
5- 5-49	0.10	0.62	neg.	0	0.9	111	2.2	5.78					
11- 3-49	0.09	0.58	neg.	0	1.1	100	2.9	6.28					
1- 5-50	0.09	0.60	neg.	0	1.4	107	1.9	5.98					
1-31-50	0.09	0.68	neg.	±	1.0	110	2.6	6.15					
3-28-50	0.06	0.52	neg.	0	0.9	100	3.6	6.37					
9- 7-50	0.06	0.58	+2	neg.	0.9	89	3.6	14.69	645	66			
11- 9-50	0.14	0.52	neg.	neg.	1.8	105	2.9	13.70					
12- 5-50	0.15	0.45	–	–	–	–	–	11.69					
12-26-50	0.21	0.40	–	–	–	–	–	10.97					
1-22-51	0.12	0.42	neg.	neg.	0.6	116	3.2	13.43	596	67	388	137	1385
3-12-51	0.25	1.94	neg.	neg.	1.1	109	3.6	71.28	644	61	535	74	1513

Expired, 4-18-51

total cholesterol) and two groups (flocculation tests and cholesterol esters) that are primarily altered by hepatocellular disease. The other reason is that all, except the Bromsulphalein test, can be determined on the same fasting sample of serum which eliminates the complications that so frequently arise when hospital personnel have to be depended upon for the collection of samples at specified times.

The additional tests are important since their results are useful in differentiating lesions of the liver other than metastatic malignancy that may produce the combination of normal serum bilirubin and abnormal Bromsulphalein and serum phosphatase tests. Such a combination may occur in hepatitis without jaundice, in cirrhosis of the Banti type, and with liver granulomata. In a recent study of the liver in sarcoidosis,[13] we encountered two patients among twenty-four proved cases with liver involvement in whom both serum alkaline phosphatase and Bromsulphalein retention were elevated and the total serum bilirubin level was below 1 mg. per cent. In tuberculosis[14] we have found the Bromsulphalein test abnormal with or without an elevation in alkaline phosphatase. In both of these diseases, however, an elevated gamma globulin almost invariably accompanies the active stage of the disease and one or more of the other flocculation tests are often positive. In hepatic metastasis, the flocculation tests become abnormal only rarely and sporadically.

The liver profile suggested for this study has been of value especially in screening the patient with a previously resected primary malignant tumor. It is our practice to study the liver function of each patient who is to have such a resection and to repeat the profile at six-month intervals after operation, if the patient remains free of symptoms, or earlier if abdominal symptoms occur.

We have found that the serum alkaline phosphatase for any individual in good health will fluctuate within rather narrow limits, even though the range for normal levels for the Shinowara, Jones, Reinhart method is fairly wide. We believe that a patient on whom liver profile studies are being made after resection of a carcinoma should be retested at monthly intervals if the serum alkaline phosphatase rises above the usual area of fluctuation for that individual, even though the higher value may still be within the upper limit of normal for the method. Such a change can probably become more readily apparent with the Shinowara than with Bodansky's method because of the wider range of normal values obtained with the former method.

Table III presents the results obtained in the liver profile series of a 67-year-old man who had a resection of the colon for carcinoma with an end-to-end anastomosis in November, 1947. Eighteen months later, he showed normal liver function values. He refused to have a Bromsulphalein test made. These results remained normal on repeated examination during the next four months, when his serum alkaline phosphatase showed a sharp rise to more than 14

TABLE IV. LIVER METASTASIS. CARCINOMA OF COLON

♂ R. MAC AGE 50	SERUM BILIRUBIN 1 MIN.	SERUM BILIRUBIN TOTAL	CEPH. CHOL.	COLL. GOLD	THYMOL TURBIDITY UNITS	THYMOL TURBIDITY 18 HR. RATIO	GLOB.	ALK. P'TASE	SERUM LIPIDS CHOLESTEROL TOTAL	SERUM LIPIDS CHOLESTEROL E/T	SERUM LIPIDS PHOSPHO. LIPIDS	SERUM LIPIDS NEUTRAL FAT	SERUM LIPIDS TOTAL LIPIDS	B.S.P. 2 MG./KG DOSE
10-19-45	0.04	0.43	neg.	+1	2.5	-								5.5
5-17-49	*OPERATION:*		*Resection of Splenic Flexure of Colon (Followed by primary end-to-end anastomosis)*											
6-30-49	0.01	0.52	neg.	+1	3.1	87	3.7	5.56						3.0
9-22-49	0.09	0.42	neg.	+1	5.2	94	3.7	5.02						
11-17-49			neg.	±	1.9	105	5.0	9.25						5.0
11-28-49	*OPERATION:*		*Further Resection as Above—*		*Recurrent Ca. of Flexure, Metastasis to Liver.*									
1- 3-50	0.19	0.86	+2	±	2.9	100	3.6	10.9						2.2
1-25-50	0.12	0.39	±	+1	2.5	100	4.0	9.24						4.1
2-21-50	0.06	0.36	+2	+1	3.5	60	4.5	8.57						5.0
4- 6-50	0.04	0.40	neg.	±	3.0	63	5.6	9.96						9.4
6-28-50	0.19	0.79	neg.	±	2.0	80	3.6	12.4						5.5
8-10-50	0.15	0.59	neg.	+.1	1.7	76	3.3	18.46	353	64				12.7
11- 9-50	0.24	1.01	+2	+1	3.6	94	3.6	29.98	247	55	228	176	741	18.8
12- 7-50	0.19	1.15	+1	±	2.9	86	5.4	29.71	310	61	268	168	873	19.9
12-14-50	0.18	0.91	+2	±	3.1	84	5.5	28.25	318	59	262	256	876	18.8

Expired, 2-18-51

units. At this time, the liver was not palpable and there was no basis upon which liver metastasis could be suspected clinically. The phosphatase remained elevated throughout the course of the disease without an increase of serum bilirubin to above normal levels until a short time before death. The flocculation tests all remained normal, with the one exception of a plus 2 cephalin cholesterol flocculation when the phosphatase was found elevated for the first time. Irregular liver enlargement was first detected on Dec. 5, 1950, three months after metastasis to the liver was suspected from the elevation of the serum alkaline phosphatase.

The results in Table III (May 5, 1949, to March 28, 1950) also illustrate the relatively narrow limits within which this patient's serum alkaline phosphatase normally fluctuated (5.78 to 6.37 units) over a ten-month period. In Table IV again is illustrated the remarkable constancy of the serum alkaline phosphatase in this patient of 5.56 and 5.02 units at approximately six weeks and eighteen weeks after resection of a carcinoma of the splenic flexure of the colon. Some eight weeks later, the concentration of his phosphatase reached 9.25 units, just above the upper limit of normal for the test. The rise above approximately 6 units, however, was considered significant and a diagnosis of liver metastasis was considered, which was confirmed nine days later at operation. The phosphatase level varied very little for the next seven months and it was not until the end of that period that a definite diagnosis of such metastasis could have been made from clinical examination alone.

The results in this patient of the Bromsulphalein test for almost one year from the date of the first liver function studies were on occasion just above our upper limit of 4 per cent retention of the dye at thirty minutes; an elevation similar in degree had been found some four years before the carcinoma developed. This brings up one of the weaknesses of the Bromsulphalein test when only slight changes from the normal are registered, for here a slight indiscernible hemolysis may contribute enough to convert a high normal result to one slightly on the abnormal side. We have not yet found a reliable method for clearing the serum when contaminated by hemolysis. As a result, while we would not emphasize such slight elevations in Bromsulphalein retention, we would rely on slight elevations of the serum alkaline phosphatase above the normal level. In this regard, we cannot subscribe to the opinion of Cabello and his associates[7] that only a significant elevation of serum alkaline phosphatase is important in its application to this problem, for these authors consider elevations of this enzyme in a range of 5.1 to 10 Bodansky units of only questionable value. In this patient (Table IV) the serum alkaline phosphatase determination gave evidence of metastasis to the liver, much sooner than did the Bromsulphalein test.

Results in the patient in Table V illustrate the reverse, in which the Bromsulphalein excretion appears to have been altered before the level of alkaline phosphatase. Two weeks before this patient was operated upon for a carcinoma of the splenic flexure, Bromsulphalein retention was 7.9 per cent, while the serum alkaline phosphatase was only 4.04 units. Although at operation the surgeon

was unable to see or palpate any metastatic lesions in the liver, the possibility that metastasis was already present at this time cannot be ruled out for the reasons already discussed.

We stated previously that the changes in both tests can only be interpreted in terms of possible metastasis when the serum bilirubin level is within normal limits, for any cause other than a hemolytic process that produces a significant elevation of serum bilirubin could also interfere with the excretion of both the dye and the enzyme in the bile. The results of the cephalin-cholesterol, colloidal gold, thymol turbidity, and gamma globulin flocculation tests were not significantly modified by hepatic metastases. We observed only an occasional plus 2 cephalin cholesterol flocculation test and gamma globulin became elevated in these patients only after the serum bilirubin levels became elevated above normal.

TABLE V. LIVER METASTASIS. CARCINOMA OF SPLENIC FLEXURE

♂ B.G. AGE 65	SERUM BILIRUBIN		CEPH. CHOL.	COLL. GOLD	THYMOL TURBIDITY		γ GLOB.	ALK. P'TASE	CHOLESTEROL		B.S.P. 2 MG./KG. DOSE
	1 MIN.	TOTAL			UNITS	18 HR. RATIO			TOTAL	E/T	
5-13-47	0.12	1.03	Neg.	±	1.1	118	-	4.04	241	48	7.9
5-27-47	*OPERATION:*				*Resection of splenic flexure—end-to-end anastomosis*						
10-14-47	0.18	1.28	Neg.	±	1.9	105	-	7.79	194	52	13.1
1- 6-48	-	-	-	-	-	-	-	11.9	-	-	-
1-13-48	0.18	1.00	Neg.	+1	1.5	100	-	9.09	204	54	10.4
3-23-48	0.18	1.43	±	+1	1.2	117	-	14.5	-	-	8.7
4-27-48	0.12	1.13	Neg.	+1	1.4	100	-	18.7	224	59	7.9
5- 4-48	0.13	1.00	Neg.	±	1.6	106	-	21.6	-	-	-
5-11-48	0.15	0.97	Neg.	±	1.0	130	6.7	18.4	204	61	-
8- 5-48	0.45	1.67	Neg.	+1	2.7	96	9.6	27.5	-	-	-
8-10-48	0.37	1.15	+1	+1	2.4	104	9.8	31.0	212	54	-
9- 9-48	0.36	1.52	Neg.	+1	2.2	100	11.3	34.6	215	47	-
9-30-48	0.68	2.14	Neg.	+1	2.4	100	11.8	32.9	237	54	-

Expired February, 1949

While a final explanation for the positive findings described cannot be given readily, certain observations at least justify some speculation about a possible mechanism. The metastatic lesions may, by compression or involvement of the intrahepatic bile passages, interfere with the excretion of the phosphatase in the bile. It has been repeatedly shown that the level of serum alkaline phosphatase is a very sensitive indicator for any interference with the flow of bile in the biliary tree.[12, 15] Furthermore, the liver forming its own phosphatase may be stimulated by the neoplastic lesions present to overproduce phosphatase even if it be only in those cells immediately adjacent to the lesions. We have observed in our study of the liver in sarcoidosis[13] that the four patients in whom we found elevated serum phosphatase levels were the only ones in whose liver tissue we found increased amounts of alkaline phosphatase histochemically in the vascular endothelium surrounding the granulomatous lesions. Also, Kabat and Furth[16] found a large amount of phosphatase histologically in the endothelial cells between the newly formed carcinomatous ductlike structures in rats, while those of the sinuses of the relatively normal liver tissue contained none or only traces.

SUMMARY

The need to suspect metastatic malignant liver disease in an individual with a normal serum bilirubin and elevated serum alkaline phosphatase is indicated, especially if accompanied by an elevated Bromsulphalein retention. Rarely, gallstone disease may produce similar results. Granulomatous involvement of the liver, however, can produce such findings but will frequently also alter one or more of the flocculation tests as well, while metastatic carcinoma will do so only occasionally and sporadically.

As routine procedure, when dealing with known malignancy, positive results with tests recommended should help in determining the probable operability of a given case. In the patient who has had a resection of a primary carcinoma, these results should clarify the significance of vague and poorly definable symptoms that appear so frequently in metastatic carcinoma.

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STUDIES ON THE THERMOGENIC RESPONSE TO INTRAVENOUS FAT EMULSIONS

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INTRODUCTION

IN THE past decade a considerable amount of research has been devoted to the development of a fat emulsion suitable for intravenous use. Despite considerable progress in this direction, many problems, particularly those concerning the untoward side reactions of the organism to the infused fat, remain unsolved. Of these, the thermogenic response following intravenous administration of fat emulsion occurs with a sufficiently high incidence to present an imposing obstacle to the general clinical use of these substances. Previous reports by Lambert, Miller, and Frost,[1] Gorens and associates,[2] Shafiroff and colleagues,[3] Meng,[4] Johnson, Freeman, and Meyer,[5] and others[6] have taken note of this problem and have advanced various hypotheses to explain its occurrence. In general, these observers have eliminated bacterial contamination and bacterial pyrogens as causes. The chemical and physical composition of emulsions, however, has received little attention. This report deals with a study of the influences of certain of these factors on the temperature response.

EXPERIMENTAL METHODS AND PROCEDURES

A. *Studies of Chemical Changes.—*

1. *Correlation between free fatty acid concentration and thermogenicity:* Emulsions of 2½ per cent corn oil (Mazola), 1 per cent Tween 60,* and 5 per cent glucose in water were prepared in a Waring Blendor one-half hour prior to intravenous administration. The emulsions were moderately stable on standing. Particle size, as measured by an ocular micrometer attached to the usual dark-field microscope, was 1 to 2 micra. The corn oil used in the emulsion given the test group of twenty-four rabbits was pretreated by solution in an equal volume of petroleum ether and passage over an alumina column, for the purpose of lowering the free fatty acid concentration. The level of free fatty acids of this and the nontreated oil was determined by titration of the oil in an isopropyl alcoholic solution, with 0.02 N sodium hydroxide, using phenolphthalein as an indicator. Carbon dioxide-free air was bubbled through the solution during titration. In this and all other tests recorded in this report, the dose of emulsion was 10 ml. per kilogram of body weight, and the technique employed was that outlined for the Standard U.S.P. XIII pyrogen test, except that the measure of thermogenicity was taken as the highest rise above the control temperature occurring during the three hours of the test. The Tween 60 and glucose were separately tested and found to be nonpyrogenic.

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*Atlas Powder Company, Polyexyethylene Sorbitan Monostearate.

2. *Effect of autoclaving:* In previous observations by Meng,[4] and later by Frost[7] and his group, it was noted that reautoclaving a known thermogenic emulsion reduced the febrile reaction. These investigators used short periods of autoclaving. We found that autoclaving a 10 per cent sesame oil emulsion* for three hours at fifteen pounds pressure provided us with a method of reducing the peroxide level to almost zero, and thus an opportunity to study the effects of this change on the thermogenic response. The method of Hills and Thiele[8] was used for peroxide determination, the oil being extracted from the emulsion after the addition of 25 Gm. of ammonium sulfate to 50 ml. of emulsion. Three extractions with 10 ml. of petroleum ether were then carried out, after which the solvent was evaporated on a steam bath. In addition, it was noted that autoclaving produced moderate increases in fatty acid concentration, monocarbonyl levels, and particle size. Pyrogen tests were carried out on a group of eight rabbits given autoclaved emulsion and compared to a control group which received unautoclaved emulsion.

B. *Studies on Physical Alteration of Emulsion. Effect of Shaking.*—In experiments designed to study the effect on thermogenicity of alteration only of particle size, batches of 10 per cent Abbott emulsions were subjected to shaking in a Fisher shaker at 250 to 280 cycles per minute for periods ranging from five to 180 minutes. Because of procurement difficulties, three separate batches of emulsion were used in this study, namely, No. 293 containing 10 per cent apricot oil, and No. 307 and No. 320 containing 10 per cent sesame oil. All three preparations also contained 0.5 per cent Tween 60, and 5 per cent glucose in water. Free fatty acid, monocarbonyl, and peroxide content were not appreciably altered by the shaking procedure. Particle size was measured by dark-field microscopy before and after each individual shaking experiment. The appearance of each bottle of emulsion was recorded after the period of shaking with respect to gross "breaking" or creaming. Pyrogen tests were then carried out using the shaken material from each of the various time intervals. During the course of the pyrogen test, dark-field microscopy was performed on samples of plasma and serum drawn from animals in both the test and control groups that developed temperature rises of 2° F. or more in the first hour of the test.

C. *The Effect of Heparin on Thermogenicity.*—In previous work in this laboratory,[10] it was observed that heparin accelerated the rate of removal of infused fat emulsion from the blood. For this reason the possible effect of heparin on the thermogenic effect was studied. From 2 to 8 mg. of heparin was added to 10 ml. per kilogram doses of apricot oil emulsion and then subjected to pyrogen tests. The emulsion was examined before and after the addition of heparin, with no apparent alteration of the gross or microscopic appearance.

D. *Studies on Aging of Emulsions.*—There is general agreement among workers in this field[2, 4, 11] that the degree of thermogenicity of a given emulsion tends to increase as the emulsion ages. A batch of 10 per cent sesame oil emulsion (Abbott) was used in this study in an attempt to detect any chemical changes which might occur during aging, and their relationship to thermogenicity. Accordingly, pyrogen tests, and determination of fatty acid, monocarbonyl,[9] and peroxide[8] levels were performed on freshly prepared emulsion, and at periods of two, four, eight, and ten weeks from the date of original preparation. It was felt that this study might provide a means of substantiating the results of the previously outlined experiments dealing with the effect of alterations in these same chemical constituents.

RESULTS

A. *Chemical Changes.*—

1. *Correlation between free fatty acid content and thermogenicity:* Pretreatment of the corn oil by passage over the alumina column resulted in 73 to 92 per cent reduction in the free fatty acid concentration as determined by titration. As can be seen in Table I the thermogenic response in the group

*We are indebted to Drs. Frost, Lambert, and Miller of the Abbott Laboratories for supplying the emulsion and performing repeated tests showing absence of bacterial contamination.

given treated corn oil emulsion did not differ significantly from that of the control group, despite the rather marked reduction in free fatty acid content.

TABLE I. RELATIONSHIP BETWEEN FATTY ACID CONTENT AND THERMOGENIC RESPONSE

	TEST GROUP. EMULSION MADE WITH ALUMINA-TREATED OIL	CONTROL GROUP. EMULSION MADE WITH UNTREATED OIL
Number of rabbits	24	24
Mean control, preinjection temp. °F.	103.3	103.4
S.D.	0.48	0.54
Mean maximal temperature rise °F.	1.23	1.49
S.D.	0.85	0.70
Mean fatty acid content of oil, meq./L.	0.62	2.31

Difference between mean temperature rise of control and test animals = 0.26; t = 1.15; P > 0.05.

2. *Effect of autoclaving:* It may be noted in Table II that our experience did not confirm previous observations concerning a reduction of the thermogenic response after reautoclaving. On the contrary, the test group which received the autoclaved emulsion exhibited a higher mean temperature rise than the control group, although this was not statistically significant. Attention is called to the fact that while the peroxide levels were reduced to almost zero, moderate increases in monocarbonyl, fatty acid, and particle size occurred. This experiment showed that a marked reduction in peroxide failed to suppress the fever reaction and, in effect, eliminated this factor as a principal cause.

TABLE II. EFFECT OF AUTOCLAVING ON THERMOGENICITY OF FAT EMULSION

	CONTROL GROUP GIVEN UNAUTOCLAVED EMULSION	TEST GROUP GIVEN AUTOCLAVED EMULSION
Number of rabbits	8	8
Mean control, preinjection temperature, °F.	103.4	103.6
S.D.	0.48	0.36
Mean maximal temperature rise, °F.	2.59	3.23
S.D.	1.39	0.54
Mean titratable acidity of emulsion, meq./L.	2.05	3.10
Mean monocarbonyl value micromols/kg.	2.36	4.21
Mean peroxide concentration, meq./kg.	177.0	0.38
Particle size, microns	1	2-4

Difference between mean temperature rise of control and test animls = −0.64; t = 1.216; P < 0.05. The minus sign (−) indicates that mean rise of test group was larger than value for control group.

B. *Studies on Physical Alteration of the Emulsion. Effect of Shaking.*— Initial observations indicated a direct relationship between the duration of shaking and the size of the resultant particles up to and including the forty-five-minute period. During this time interval, particle sizes were in the range of 3 to 25 micra, and it was found that further shaking for ninety and 180 minutes did not cause changes of the same magnitude as were observed when the duration was increased from five to forty-five minutes. Occasionally particles as large as 40 to 50 micra were found in the ninety- and 180-minute periods, however, which were not present in the shorter durations of shaking. As previously indicated, three batches of emulsion were used in this study, and this

fact is of some importance with respect to our observations, because there was an indication that the older the emulsion (from the date of original preparation) the more readily it would break on shaking. When the results on all three batches are combined as in Table III, it will be noted that emulsion shaken for five, ten, and twenty minutes produced temperature rises which were slightly higher than in the control group. In the groups given emulsion shaken for forty-five to 180 minutes, this trend is reversed and a decrease in thermogenicity occurred. This decrease was most prominent in the ninety- and 180-minute groups in which all of the animals exhibited a short but variable period of a shocklike state immediately after administration. Rapid, shallow respiration was a constant finding during this period. Five of the twenty-four rabbits died within twenty-four hours, autopsies revealing multiple petechiae in both lungs, pulmonary edema, and pleural effusion. Only one of the animals in the forty-five-minute group died, with findings identical to those described previously. Histologic examination of the lungs with both hematoxylin and eosin, and oil-red-O stains corroborated the gross impression of fat trapping and embolization.

TABLE III. EFFECT OF DURATION OF SHAKING FAT EMULSION* ON THERMOGENIC RESPONSE

DURATION OF SHAKING, MINUTES	NO. OF RABBITS IN EACH GROUP	MEAN HIGHEST TEMPERATURE RISE		GROSS APPEARANCE	MICROSCOPIC APPEARANCE, PARTICLE SIZE (MICRA)
		SHAKEN °F.	UNSHAKEN °F.		
5	12	2.25	1.90	Broken ±	1-5
10	24	1.92	1.80	Broken ±	3-7
20	24	1.89	1.20	Broken	3-10-15
45	24	1.4	1.83	Broken	3-25
90	12	1.3	2.0	Broken	3-25-35
180	12	0.61	2.21	Broken	3-40

*Composite results of all batches of emulsion used.

Table IV presents data showing that it was not possible to correlate the size of the particle injected with the particle size subsequently appearing in the serum or plasma, despite the development of considerable temperature elevation.

TABLE IV. PARTICLE SIZE OF FAT IN SERUM AFTER INTRAVENOUS INJECTION OF FAT EMULSION (CORRELATION WITH THERMOGENICITY)

	PARTICLE SIZE INJECTED (MICRA)	MAXIMUM TEMPERATURE RISE (°F.)	PARTICLE SIZE IN PLASMA (MICRA)
Control emulsion	1-2	2.4	1-2
Shaken emulsion			
Five minutes	1-5	2.2	1-2
Twenty minutes	3-10	2.8	1-2
Forty-five minutes	3-25	2.8	1-2

C. *Effect of Heparin on Thermogenicity.*—The addition of 2 to 8 mg. of heparin to 10 ml. per kilogram doses of 10 per cent apricot oil emulsion caused alteration in neither gross nor microscopic appearance of the preparation. Graded amounts of heparin were used because in our initial studies on the

thermogenic effect of heparin alone, two of four rabbits died four hours after receiving 5 mg. per kilogram intravenously. Autopsy failed to disclose the cause of death. As shown in Table V, no appreciable change in the magnitude of the thermogenic response was noted in the sixteen test animals receiving the emulsion plus heparin. Pyrogen tests using the same batch of heparin in 5 mg. per kilogram amounts without fat emulsion were found to be negative. None of the animals in this series died.

TABLE V. EFFECT OF HEPARIN ON THE THERMOGENIC RESPONSE

	CONTROL GROUP GIVEN 10 PER CENT APRICOT OIL EMULSION	TEST GROUP GIVEN 10 PER CENT APRICOT OIL EMULSION PLUS HEPARIN (2-8 MG./KG.)
Number of rabbits in each group	16	16
Mean control, preinjection temperature, °F.	103.3	103.6
S.D.	0.44	0.41
Mean maximal temperature rise, °F.	1.61	1.75
S.D.	0.82	0.71

Difference between mean temperature rise of control and test animals = −0.14; t = 0.5150; P > 0.05. The minus sign (-) indicates that mean temperature rise of test group was larger than value for control group.

D. *Studies on Aging of Emulsions.*—In the particular batch of 10 per cent sesame oil emulsion used in this ten-week period of observation, the results indicate (Table VI) that thermogenicity did not increase on aging despite moderate elevation in fatty acid, monocarbonyl, and peroxide concentrations. These changes are presumably a result of the oxidative and lipolytic processes which fat emulsions undergo during storage. The results of this particular experiment corroborate the findings after autoclaving and pretreatment of the oil with alumina in that thermogenicity did not vary with changes in chemical constitution.

TABLE VI. EFFECT OF AGING OF EMULSION OF THERMOGENICITY AND CHEMICAL CONSTITUENTS

AGE OF EMULSION FROM DATE OF PREPARATION	NUMBER OF RABBITS	CONTROL TEMPERATURE		MAXIMAL TEMPERATURE RISE		AVERAGE FATTY ACID CONTENT (MEQ./L.)	AVERAGE CARBONYL VALUES (MICROMOLS/KG.)	AVERAGE PEROXIDE VALUES (MEQ./KG.)
		MEAN °F.	S.D. °F.	MEAN °F.	S.D. °F.			
Fresh—1 week	16	103.4	0.39	1.31	0.75	1.76	1.16	17.5
2 weeks	8	103.2	0.41	1.48	0.67	1.49	0.83	17.5
4 weeks	22	103.4	0.50	1.92	0.63	1.12	1.23	32.4
8-10 weeks	16	103.6	0.38	1.54	0.75	2.30	1.60	29.6

DISCUSSION

Despite general asknowledgment of the importance of this problem, the actual cause of the fever response remains obscure. Shafiroff[3] advanced the theory that rapid oxidation of the infused fat could account for a temperature rise; this idea is neither supported by experimental evidence, however, nor does it comply with established principles of heat regulation and energy utilization and production. Lambert and his co-workers[1] expressed the opinion that the thermogenic response is an inherent property of fat emulsions, but

they later were able to prepare some batches of fat emulsion which were not thermogenic.[7] Neptune and co-workers[6] felt that pyrogens present in commercial oils may be the responsible agents, while Johnson, Freeman, and Meyer[5] attributed thermogenic properties to certain emulsifiers which have been used. Creditor[12] suggested that hemolysis produced by intravenous fat infusion might be involved in the thermogenic response. In all of these reports, as well as the work presented here, bacterial contamination has been eliminated as the cause. The work of Lambert and his group[1] indicates that bacterial pyrogens in the oils used in making the emulsions are not responsible for the thermogenicity of these preparations. There has been general agreement with the observations that aging of the emulsions beyond three to four weeks usually enhances thermogenic properties, although in the particular preparation which we studied this was not the case.

With the exception of Lambert's attempt to prepare a pyrogen-free emulsion with oil of low peroxide content, to our knowledge, no previous study has attempted to correlate the occurrence of the fever response with specific chemical and physical changes which may occur in the emulsions. Our studies indicate that neither alteration of the particle size nor certain chemical changes such as peroxide, monocarbonyl, or fatty acid content can be clearly correlated with the magnitude of the thermogenic response. Despite this, the possibility remains that chemical or physical changes other than the ones studied by us may prove to be correlated with the fever reaction.

The effect which a short period of shaking exerted on particulate size of the emulsions presents obvious practical implications with respect to transporting this material to installations which may be a considerable distance from the site of preparation.

The accumulation of fat in the lungs of laboratory animals following intravenous infusion of both stable and unstable fat emulsions has been well authenticated.[13, 14] Only when relatively unstable emulsions are used is this pulmonary trapping of fat accompanied by signs and symptoms of pulmonary embolism. This was true of our own experience in rabbits given emulsion shaken for longer periods of time. These animals developed labored breathing, variable degrees of shock, and occasionally convulsions followed by death. Of the entire group, however, only five of the animals died; the remainder recovered from the initial shocklike state, and developed some degree of temperature elevation. Previous work in this laboratory on intravenous infusion of corn oil emulsions in rats, with examination of the lungs at increasing time intervals, demonstrated considerable pulmonary trapping of fat with subsequent complete clearing of the pulmonary bed. These observations suggested the possibility that small pulmonary emboli, or trapping of fat particles in the pulmonary capillary tree of a degree insufficient to produce impairment of blood circulation, might produce the thermogenic reaction.

The tendency for the thermogenic response to be higher when the emulsion had been shaken for short periods, as observed in the present study, supports the possibility that pulmonary trapping may be concerned. This effect did not occur consistently enough, however, to permit firm conclusions to be

drawn. One may speculate that short-term shaking would increase particle size and decrease stability of the emulsion in plasma, leading to increased pulmonary trapping, but that further increases in particle size and instability of the emulsion would lead to frank pulmonary embolism attended by circulatory impairment which would suppress the thermogenic response. The failure to find a correlation between the particle size of fat globules in the plasma and the thermogenic response does not necessarily argue against the pulmonary trapping hypothesis because larger particles would probably be filtered out by the lungs in each circuit of blood through them. Despite the negative nature of the present data, we feel that this problem is of sufficient import to warrant further attempts to correlate thermogenicity with other physical and chemical changes in emulsions.

SUMMARY AND CONCLUSIONS

Experiments on rabbits were conducted to ascertain the possible effect of chemical and physical changes which occur in fat emulsions on the production of the thermogenic response.

These studies indicate that neither alteration of particle size, nor certain chemical changes such as peroxide, monocarbonyl, and fatty acid concentration, can be clearly correlated with the magnitude of the thermogenic response.

Observations made during the course of this study suggest that pulmonary trapping of fat requires further study as a possible factor in the causation of the febrile response.

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SEROTONIN ACTIVITY IN BLOOD

Measurements in Normal Subjects and in Patients with Thrombocythemia, Hemorrhagica, and Other Hemorrhagic States

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RAPPOPORT in 1948 proposed the name "serotonin" for a vasoconstrictor substance which he had isolated in crystalline form from blood serum. Subsequent chemical analysis showed it to be 5-hydroxytryptamine creatinine sulfate; synthesis of this chemical yielded a product with properties identical to those of the original crystalline material, and 5-hydroxytryptamine proved to be the active moiety of the molecule.[1-6]

The existence of a vasoconstrictor material in blood serum was first inferred by Ludwig[7] when he encountered unexpected resistance to perfusion of dog muscle with defibrinated blood. Epinephrine as the active serum principle was excluded by the observation of O'Connor[8] that the vasoconstrictor property was absent in plasma prior to clotting and by his demonstration that pigeon gut was constricted by serum and relaxed by epinephrine. Janeway[9] confirmed the observation that there was little vasoconstrictor activity in plasma and found that platelets were necessary for its appearance in serum. The active principle was dialyzable and heat stable. Hirose[10] found the sera of thrombocytopenic patients to be relatively poor in vasoconstrictor activity. In 1942, Reid[11] again showed that a vasoconstrictor substance appeared in the blood following coagulation and that platelets were necessary for its production. Subsequently Rand,[12] using Rappoport's isolation procedure for serotonin, demonstrated that serum derived from platelet-rich bovine plasma was rich in 5-hydroxytryptamine creatinine sulfate, while the parent plasma was almost devoid of this substance.

Thus it is now clear that serotonin, a simple chemical substance with vasoconstrictor properties, arises in the blood during clotting in the presence of platelets. Zucker[13] has concluded that a vasoconstrictor material liberated by platelets at the site of vascular injury contributes to hemostasis in the rat and Correll[14] has shown that parenterally administered serotonin retards bleeding from incised wounds in rats, mice, guinea pigs, and chickens. Although the impaired concentration of serotonin in the sera of thrombocytopenic patients has already been indicated, little is known of the serotonin concentration in other hemorrhagic states wherein the platelets are present in normal or even in increased numbers. Such a study is presented in the present paper.

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METHODS

Serotonin was measured by its contractile effect on the circular smooth muscle of bovine carotid artery.[9] Artery samples, obtained within one hour of slaughter of the animal, were placed in ice-cold balanced saline solution[15]* and brought to the laboratory. Following removal of connective tissue, 0.5 cm. segments were cut off, opened longitudinally, and affixed by sutures to a muscle lever so as to employ the contractile power of the circular muscle fibers. The muscle-strip was suspended vertically in a chamber of 4 ml. volume, the lower attachment for the strip being also the outlet for a flow of oxygen to assure mixing and oxygenation. The entire chamber was immersed in a water bath at 37.5° C. The muscle lever, adapted from descriptions of Janeway[9] and Meyer,[16] inscribed a tracing on a constant-speed kymograph drum. The paper speed was about 0.6 mm. per minute, and alterations of muscle length were seven times magnified in the tracing by reason of the unequal lengths of the lever arms.

The muscle-strip was prepared for use by covering it with balanced saline solution in the chamber and applying a preliminary stretching force of 45 Gm. for thirty minutes. On removing this force, the muscle-strip retracted a variable amount and usually came to a steady state in about twenty minutes. The balanced saline solution or other test fluids bathing the muscle-strip were introduced into the chamber or removed therefrom with capillary pipettes. When the muscle-strip had reached a steady state, observations were begun by rapidly removing the balanced saline solution from the chamber and substituting a test fluid. After exposure to the muscle-strip for five minutes, the test fluid was removed, balanced saline solution replaced in the chamber, and the muscle-strip permitted to relax and recover from any preceding contraction. Repetition of such cycles was sometimes possible up to ten times with certain muscle-strips. Other strips, however, proved to be inert at the outset, or to become so rapidly for obscure reasons. Therefore, in contrasting the vasoconstrictor potency of two test fluids, only if the second of the two responses exceeded the first was it possible to conclude that the second test fluid was more vasoconstrictor than the first. In some cases, indication of the unaltered sensitivity of the muscle-strip was obtained by bracketing the response to one test fluid between two responses to another test fluid.

During the course of repeated tests, a slowly rising base line was often observed, or there was incomplete relaxation from preceding contractions. In most of the observations, therefore, the base line was artificially rectified between tests by readjustment of the apparatus.

Clinical tests of hemostasis and coagulation included measurements of bleeding time by the method of Duke[7] and of capillary fragility by constriction of the arm for ten minutes by a cuff set at 100 mm. Hg. Platelets were counted directly in counting chambers. Plasma and serum prothrombin were estimated with a one-stage procedure, employing as diluent barium sulfate-treated prothrombin-free normal plasma.[18] Serum prothrombin conversion accelerator (SPCA) was measured by the method of Alexander.[19] Clot retraction was semiquantitated by the method of Aggeler.[20] Antihemophiliac potency and circulating anticoagulant were examined by means of the recalcification time determined according to Quick[21] with the modification of adjusting the pH to 7.35 by equilibration with a gas mixture of 5 per cent carbon dioxide and 95 per cent oxygen before recalcifying. The recalcification time of mixtures containing 20 per cent patient plasma and 80 per cent normal or hemophiliac plasma was contrasted with the recalcification time when normal plasma replaced that of the patient.

RESULTS

Observations of Serotonin Evolution in Normal Blood.—The reliability of the muscle-strip as an indicator of vasoconstrictor potency was demonstrated by the reproducibility of the response to repeated applications of the same

*The balanced saline solution contained per liter of aqueous solution: NaCl, 8.0 Gm.; KCl, 0.4 Gm.; $MgSO_4 \cdot 7H_2O$, 0.08 Gm.; $MgCl_2 \cdot 6H_2O$, 0.08 Gm.; $CaCl_2$, 0.14 Gm.; $Na_2HPO_4 \cdot 2H_2O$, 0.06 Gm.; KH_2PO_4, 0.06 Gm.; glucose, 1.0 Gm.; phenol red, 0.02 Gm.; penicillin and streptomycin, 50,000 units each; and $NaHCO_3$ sufficient to bring the pH to 7.2 to 7.4.

stimulus. In Fig. 1 are shown three very similar responses to three applications of normal serum diluted 1:6 with balanced saline solution, followed by a fourth and larger response to undiluted normal serum. The similarity of

Fig. 1.—Three submaximal responses of the muscle-strip to repeated application of diluted normal serum followed by a fourth and greater response to undiluted normal serum.

In this and in all the other figures, the short vertical marks on the straight horizontal line indicate the application of reagents to the muscle-strip. In all sequences, the first line indicates the removal of balanced saline solution and introduction of a test fluid, which after five minutes was replaced at the second line by balanced saline solution. Twenty minutes later, at the third line, a second test substance was introduced and the cycle repeated.

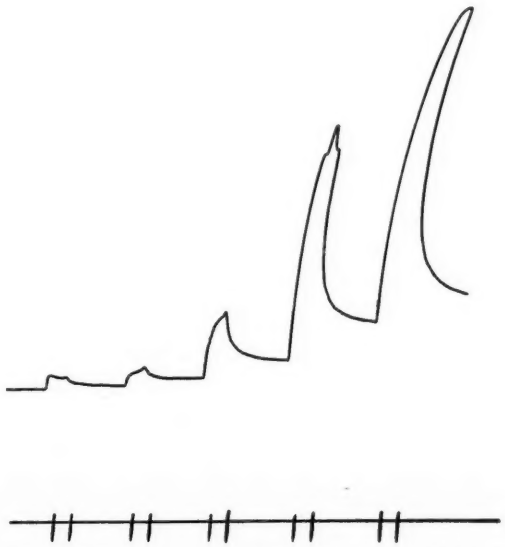

Fig. 2.—Graded responses of the muscle-strip to normal serum diluted 1:16, 1:8, 1:4, and 1:2 with balanced saline solution and to undiluted normal serum.

these submaximal responses to the same stimulus permits the conclusion that the second of two responses, if it were the greater, was caused by a greater stimulus. In Fig. 2 a series of dosage-response curves is presented. These were obtained with normal serum diluted 1:16, 1:8, 1:4, and 1:2 with balanced

saline solution, and finally with undiluted normal serum. Undiluted normal serum was approximately equal in vasoconstrictor potency to a solution of synthetic serotonin* in balanced saline solution containing 0.5 μg per ml. In accordance with Janeway's observations,[9] it was found that the vasoconstrictor principle was reduced in serum derived from normal plasma that had been made platelet-poor before coagulation. The vasoconstrictor principle easily penetrated a dialysis membrane, was stable in frozen serum for many weeks, and was nearly absent in unclotted normal plasma.

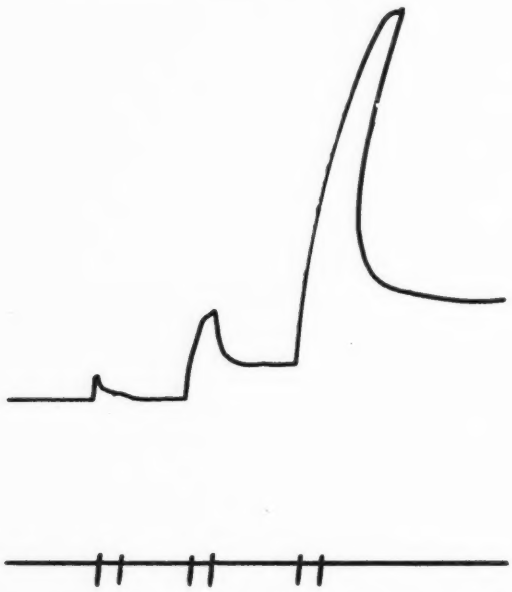

Fig. 3.—Three responses of the muscle-strip induced in sequence by heparinized balanced saline solution, by heparinized plasma rich in intact platelets, and by heparinized platelet-rich plasma in which the platelets had been fragmented by freezing and thawing.

In order to clarify the part played by platelet-rupture in the evolution of serotonin, the effects on the muscle-strip of uncoagulated platelet-rich plasma were contrasted before and after freezing of the plasma at −70° C. Heparin was chosen as the anticoagulant because of the observation of Rand[22] that heparin had little direct effect on the muscle-strip. Citrate might have been used, but oxalate and the disodium salt of ethylenediaminetetraäcetic acid (Sequestrene) were both intrinsically vasoconstrictor. Since oxalated balanced saline solution constricted the muscle-strip while balanced saline solution devoid of calcium had no such effect, it seemed probable that the free oxalate ion rather than the lack of calcium was the constrictor agent in oxalated plasma. In Fig. 3, it is shown that heparinized balanced saline solution was feebly constrictor while heparinized platelet-rich plasma, prior to freezing, was only slightly more potent. After thrice being frozen and thawed, the same heparinized platelet-rich plasma evinced greatly enhanced vasocon-

*Crystalline samples of serotonin creatinine sulfate were kindly supplied by Dr. K. E. Hamlin, Abbott Laboratories, North Chicago, Ill., and by Dr. M. E. Speeter, The Upjohn Company, Kalamazoo, Mich.

strictor activity. The platelets, examined by phase-contrast microscopy, were much fragmented in this frozen sample, in contrast to their intact state in the unfrozen platelet-rich plasma. Since plasma devoid of platelets was not found to gain in vasoconstrictor potency on repeated freezing, it seemed evident that mechanical fragmentation of platelets in plasma liberated serotonin. Thus neither conversion of prothrombin to thrombin nor coagulation of fibrinogen was essential for this result in vitro.

Serotonin has been reported to influence neither the conversion of prothrombin to thrombin nor the reaction between thrombin and fibrinogen.[14] In the present study, commercial thromboplastin* and thrombin† were found to be devoid of vasoconstrictor activity, while crystalline serotonin failed to rectify either the prolonged recalcification time of platelet-poor plasma or the defective clot retraction and prothrombin consumption observed when such plasma was clotted by recalcification.

Since intact platelets become altered in appearance or fragmented during normal coagulation,[23] it is possible that these changes are necessary for serotonin production. In order to investigate the stage of the coagulation cycle at which the intact platelet was associated with serotonin evolution, samples of platelet-rich oxalated plasma were defibrinated either by recalcification or by addition of bovine thrombin. The resulting platelet-free sera were found to be of equal serotonin content. Although the serum obtained by thrombin-defibrination was recalcified prior to the test in order to remove oxalate, the resulting conversion of prothrombin to thrombin in this sample could have had no further influence on intact platelets since they had already been removed by the thrombin-induced clot. Thus the appearance of serotonin from the intact platelet seemed independent of events of the clotting cycle prior to the evolution of thrombin.

Because heparinized plasma containing intact platelets had little serotonin activity, an attempt was made to produce an equally inert suspension of washed platelets in balanced saline solution. All such preparations, however, showed potent vasoconstrictor action, although the platelets appeared intact following the washing and suspending procedure. Deliberate fragmentation of these platelets by freezing did not further increase the potency of the sample. Probably unapparent platelet damage had occurred, sufficient to liberate serotonin either directly from the platelets, or by interaction of a platelet factor with traces of plasma still adhering to the platelets.

Observations of Serotonin Evolution in the Blood of Patients With Hemorrhagic States.—Diminished serotonin content was observed in the sera of all of nine thrombocytopenic subjects whose platelets numbered less than 100,000 per cubic millimeter. The group included four patients with idiopathic thrombocytopenic purpura, one of whom was in hematologic relapse following splenectomy, while the other three patients were examined before splenectomy. A rise in platelet count to normal levels in one of these subjects following splenec-

*Extract of Difco thromboplastin, diluted 1:20 with balanced saline solution.
†Parke, Davis bovine thrombin, 5 units per milliliter in balanced saline solution.

tomy was associated with restitution of serotonin concentration to normal. This sequence is illustrated in Fig. 4, which contrasts the serotonin activity before splenectomy, when the whole blood platelet count was 10,000 per cubic millimeter with the serotonin concentration following splenectomy when the platelet count was 200,000 per cubic millimeter. The patients with idiopathic thrombocytopenic purpura exhibited increased capillary fragility, prolonged bleeding time, diminished clot retraction and prothrombin consumption, and normal prothrombin concentration. The remainder of the group of patients with thrombocytopenic purpura included four individuals with diffuse liver disease, and one patient with lymphatic leukemia. These patients with secondary thrombocytopenic purpura additionally exhibited decreased prothrombin concentration, which averaged 47 per cent of normal for the group.

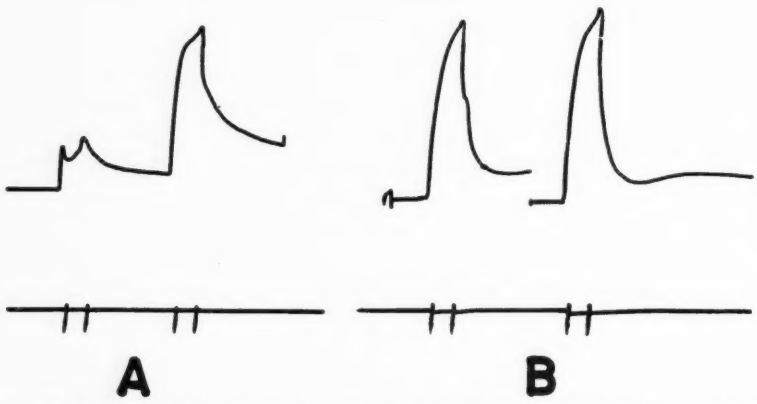

Fig. 4.—The muscle-strip response to the serum of a thrombocytopenic subject followed by the response to normal serum: *A*, Before splenectomy, whole blood platelet count 10,000 per cubic millimeter. *B*, After splenectomy, whole blood platelet count 200,000 per cubic millimeter.

Impaired serotonin despite normal platelet numbers was observed in six hypoprothrombinemic patients. The hypoprothrombinemia was associated with cirrhosis in two instances and once each with polycythemia with splenomegaly, multiple myeloma, and uremia. In one instance no cause for hypoprothrombinemia could be found. Normal serotonin and platelet concentrations coexisted, however, in four other hypoprothrombinemic subjects. These patients were respectively affected with diabetes with uremia, disseminated lupus erythematosus, cirrhosis, and refractory anemia with hepatosplenomegaly. The minimal prothrombin concentration in this group with normal serotonin was 9 per cent in the patient with cirrhosis. Diminished serotonin was not, therefore, a necessary concomitant of hypoprothrombinemia. Clinical bleeding, manifested by purpura, bruises, or melena, was noted in three of the six hypoprothrombinemic patients with decreased serotonin and normal platelet numbers, and in two of the four similar subjects with normal serotonin. The remaining hypoprothrombinemic patients showed no spontaneous bleeding.

Three hemophilic subjects were examined for serotonin evolution. All three presented laboratory tests characteristic of the disease and all had fre-

quently been observed at this laboratory during bleeding episodes. Two of the three offered the history of a bleeding tendency among males of the maternal branch of the family. One of the three hemophilic patients showed a potent circulating anticoagulant and strongly resisted the usual clot-promoting effects of fresh plasma both in vivo and in vitro. This individual repeatedly exhibited subnormal serotonin, without regard to how long his blood was permitted to clot before testing the serum. The remaining uncomplicated hemophilic patients showed a decreased rate of serotonin evolution which seemed to parallel the delay in clotting, but the final concentration closely approximated that of the normal.

No impairment of serotonin was found in the serum of a 15-year-old girl with a bleeding tendency that might best be called pseudohemophilia, as described by von Willebrand and by Jürgens[24, 25] under the title of "constitutional thrombopathy" and by Alexander as "pseudohemophilia."[26] The patient had experienced epistaxis, bleeding gums, joint hemorrhage, easy bruising, and menorrhagia. There was no familial history of bleeding. The platelets were of normal number and appearance, but capillary fragility and bleeding time were increased, and the clotting time was usually moderately prolonged. Prothrombin consumption, antihemophilic potency, and SPCA were impaired on several occasions. The abnormal nail bed capillaries described by Alexander[26] and by MacFarlane[27] were not sought for. No circulating anticoagulant was present. A second patient, probably another instance of pseudohemophilia as judged by ready bruising, menorrhagia, prolonged bleeding and clotting times, and decreased antihemophilic potency, also had a normal level of serotonin.*

In view of the uncertainty as to the exact mechanism whereby coagulation in the presence of platelets liberates serotonin, it was of interest to examine a patient with congenital afibrinogenemia. Observations were therefore performed on blood samples obtained from the patient already described by Lawson.[28]† In Fig. 5 is shown the muscle-strip response to the patient's serum bracketed between two responses to normal serum. It is apparent that normal or nearly normal serotonin evolution is possible despite absence of clotting in the usual sense. The shed blood of this patient, though remaining indefinitely fluid, nevertheless contained prothrombin in normal concentration, and conversion of prothrombin to thrombin was rapid. Thus the coagulation cycle was intact up to and including the evolution of thrombin. The patient's platelets, normal in number, were seen by means of phase-contrast microscopy to fragment in shed blood unless oxalate was added to prevent thrombin evolution.

Thrombocythemia has been reported by Mortensen[29] to be associated with a bleeding tendency of obscure nature. Blood samples of six thrombocythemic patients, all with platelet counts exceeding 1,000,000 per cubic millimeter, have been examined for their serotonin content. Three of these patients have been examined in some additional detail as follows.

*I am much indebted to Dr. Janet W. McArthur, Massachusetts General Hospital, Boston, Mass., for the opportunity to study these two patients.

†This patient was studied through the helpful arrangements of Dr. Herman A. Lawson, Veterans Administration Hospital, Providence, R. I.

CASE 1.*—A 34-year-old man was hospitalized because of hematemesis and melena. Seven years earlier, splenic enlargement had been discovered following severe trauma to the abdomen, but no symptoms had occurred until gastrointestinal bleeding began five years later. The still enlarged spleen had been removed after one year of repeated bleeding from esophageal varices. The present hospitalization, one year after the splenectomy, was occasioned by continued hematemesis and melena which had necessitated over ninety

Fig. 5.—The muscle-strip response to the serum of a subject with congenital afibrinogenemia, bracketed between two responses to normal serum.

transfusions. At this time, the liver was slightly enlarged, although tests of liver function were unimpaired with the exception of prothrombin concentrations ranging from 40 to 50 per cent of normal. Liver tissue obtained by biopsy appeared normal. The bleeding time repeatedly exceeded ten minutes. The capillary fragility, clot retraction, and prothrombin consumption were normal. The platelets numbered 4,000,000 to 6,000,000 per cubic millimeter on repeated estimations, and were not unusual as seen in the stained smear, except for occasional large forms. Although the white blood cells numbered 40,000 to 50,000 per cubic millimeter, the stained blood film was not remarkable except for a slight increase in eosinophils and basophils. A biopsy specimen of bone marrow was characterized by hyperplasia of all of the cellular elements including the megakaryocytes. The portal pressure, measured at laparotomy, was 270 mm. of saline solution. An effective shunt could not be established and the patient later succumbed. At necropsy, the splenic and portal veins were observed to be occluded by an old thrombotic process.

Repeated estimations of serotonin in Case 1 indicated a striking deficiency, as illustrated in Fig. 6. Administration of cortisone for two weeks failed to alter this pattern, although the bleeding time reverted to normal. Normal serum, diluted 4:5 with serum of Case 1, retained serotonin activity, and thus the serum of Case 1 probably contained no potent antiserotonin effect or inhibitor of arterial contractility. In an attempt to discover whether the platelets of Case 1 were unusually stable or otherwise abnormal, a sample of the platelet-rich plasma of Case 1 was exposed to sonic energy at 400 kilocycles per second. This effected extensive fragmentation of the platelets. The plasma then was

*For the opportunity to study this patient, acknowledgment is made with pleasure to Dr. Frank H. Gardner, Peter Bent Brigham Hospital, Boston, Mass., and to Dr. Daniel S. Ellis and Dr. Robert R. Linton, Massachusetts General Hospital, Boston, Mass.

defibrinated and its serotonin content contrasted with that of a similarly treated portion of normal control plasma having the same original platelet concentration. Under these conditions, serotonin activity of Case 1 more nearly approached but did not equal that of the normal control.

CASE 2.*—A 50-year-old woman was accidentally discovered to have splenic enlargement to 9 cm. below the left costal margin. The liver was 2 cm. below the right costal margin by percussion. There were no symptoms except for the appearance of evanescent

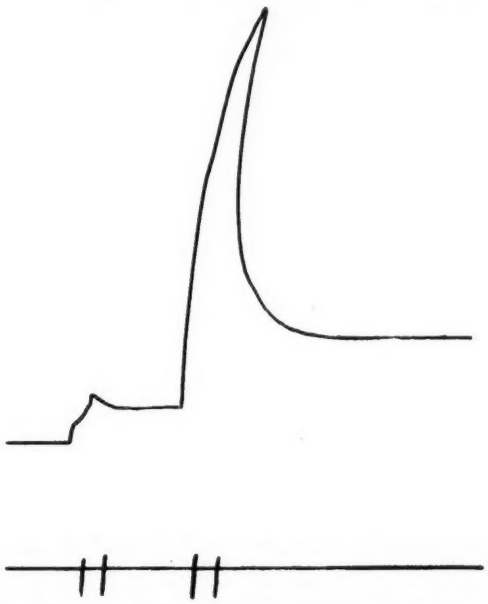

Fig. 6.—The muscle-strip response to the serum of Case 1 followed by a much greater response to normal serum.

tender nodules in the skin together with easy bruising and fatigue during the preceding three years. Sternal biopsy incision bled for thirty-six hours, and a trifling laceration of a finger bled for many hours. The marrow specimen was notable for a general hyperplasia of cellular elements and a great increase in megakaryocytes. The platelets numbered 2,839,000 per cubic millimeter, the bleeding time was increased to ten minutes, and capillary fragility was normal. Clot retraction, prothrombin consumption, coagulation time, and recalcification time were unimpaired, but the prothrombin concentration was 56 per cent. Although there was a slight leukocytosis, the differential count of the stained smear was normal. The platelets did not appear unusual aside from their excess numbers.

Serotonin potency of serum of Case 2 was diminished despite the elevation of platelet numbers. In Fig. 7 is illustrated the inadequacy of the patient's serotonin, bracketed between two normal responses.

CASE 3.—A 58-year-old man entered the hospital because of neuritic pain in the right leg. Weakness and left upper quadrant distress had been present for six months, and splenomegaly had been discovered two months before the present admission. The white blood cells then numbered 348,000 per cubic millimeter, the platelets 150,000 per cubic millimeter, and the stained smear contained a profusion of myelocytes. Splenic irradiation on the basis of a diagnosis of myelogenous leukemia sharply reduced the total white cell count.

*This patient was kindly made available for study by Dr. Frank H. Gardner, Peter Bent Brigham Hospital, Boston, Mass., and by Dr. Russell B. Street, Quincy City Hospital, Quincy, Mass.

During the present admission the spleen was found to be enlarged to the iliac crest and the liver to 5 cm. below the right costal margin. The platelets numbered 1,260,000 per cubic millimeter, and the bleeding time exceeded ten minutes on several occasions, but the capillary fragility was normal. The white blood cells, 107,000 per cubic millimeter, were predominantly myelocytes and neutrophils. The prothrombin concentration was reduced to 48 per cent of normal, but recalcification time, prothrombin consumption, and clot retraction were normal. The hospital course of the patient in Case 3 was complicated by a very extensive spontaneous hematoma involving the right upper arm, the axilla, and the chest wall.

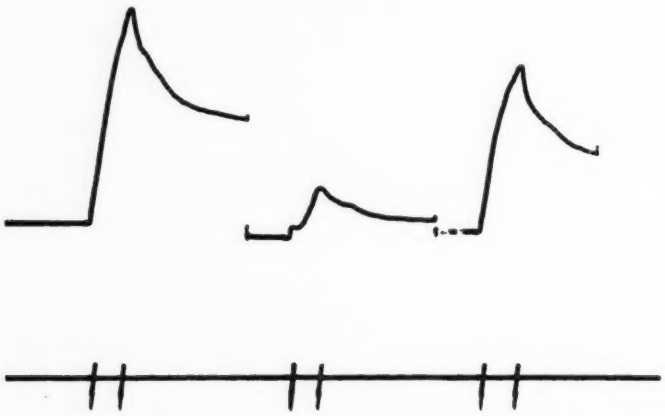

Fig. 7.—Muscle-strip response to the serum of Case 2, bracketed between two responses to normal serum.

The serotonin content of the serum of Case 3 was much diminished despite the superabundance of platelets in the whole blood. Artificial disintegration of the platelets in Case 3 failed to evolve serotonin to match the normal control, as indicated in Fig. 8. In this test, plasma of Case 3 containing 1,000,000 platelets per cubic millimeter was thrice frozen at –70° C. After defibrination by replacement of calcium, the serum was contrasted for its serotonin content with that of a serum similarly prepared from normal plasma having 500,000 platelets per cubic millimeter.

Decreased serotonin activity in the presence of thrombocythemia was observed in serum samples of three additional patients. The platelet concentrations ranged from 2,000,000 to 4,000,000 per cubic millimeter. One of the individuals* had had splenomegaly five years previously followed by splenectomy. Thereafter bleeding occurred from various sites together with a prolonged bleeding time, thrombocythemia, impaired prothrombin consumption, and slightly decreased prothrombin concentration. In this instance the patient's serotonin potency was about one-quarter of normal.

The remaining two patients also exhibited a bleeding tendency.† One had had polycythemia and splenectomy two years previously, the other was diagnosed as myeloid metaplasia at necropsy.

Normal serotonin has been observed in one thrombocythemic patient whose platelets numbered 1,700,000 per cubic millimeter. This individual bruised rather readily but had no other tendency to bleed. The white cells were increased to 15,000 per cubic millimeter, the stained smear being otherwise normal. There were no enlarged organs and capillary fragility and bleeding time were both normal.

*The data and blood samples of this patient were kindly furnished by Dr. F. W. Gunz, Public Hospital, Christchurch, New Zealand.

†Blood samples of these patients were kindly supplied by Dr. Bernard M. Jacobson, Massachusetts General Hospital, Boston, Mass., and by Dr. Frank H. Gardner, Peter Bent Brigham Hospital, Boston, Mass.

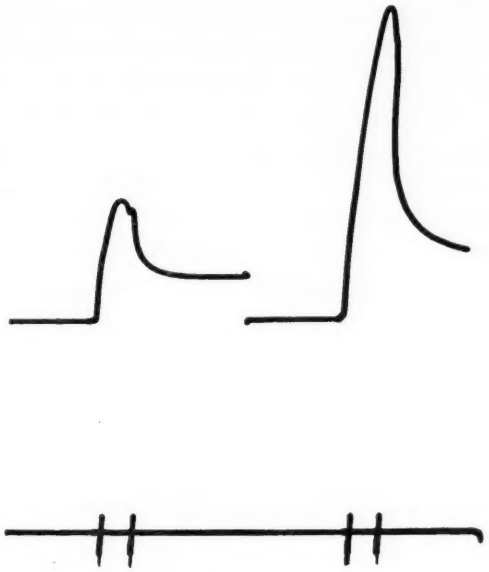

Fig. 8.—Muscle-strip response to the serum of Case 3 followed by a greater response to normal serum. Both sera were prepared from plasma samples in which the platelets (twice as numerous in Case 3 plasma as in the normal plasma) had been artificially fragmented prior to clotting.

DISCUSSION

The known action of serotonin is relatively simple, consisting of stimulation or constriction of smooth muscle. This has been shown with carotid artery of sheep and ox, jejunum of mouse and guinea pig, uterus of virgin rat, ear vessels of rabbit, bronchus of dog, and denervated nictitating membrane of cat.[2, 22, 30-33] The response of isolated smooth muscle to serotonin is potentiated by cocaine,[30] unaffected by atropine,[30] and antagonized by yohimbine[30, 34] and by ergotoxine.[34]

Given parenterally, serotonin briefly elevates the blood pressure of rabbit, dog, and man.[33, 35, 36] An initial depressor phase of vagal origin may occur which can be abolished by atropinization or vagotomy. In the cat, depression of blood pressure by this vagal mechanism is the dominant action of serotonin.[35] The pressor response is not prevented by adrenergic blocking agents[33, 35] and may be augmented by Pituitrin. Tachyphylaxis to Pitressin, however, is associated with temporary unresponsiveness of the blood pressure to serotonin. Tachyphylaxis to serotonin itself is either of only five to fifteen minutes' duration[33] or does not occur.[35] Chemical analogs of serotonin, such as tryptamine and 7-hydroxytryptamine, are inhibitory[31] or have feeble vasoconstrictor properties.[35]

Although serotonin is found in normal serum, it has also been isolated from other tissues. Chromaffin tissue of the gut is reported to be rich in this substance, as well as salivary and cutaneous glands of various species.[37, 38] In dogs most activity is present in the pyloric region of the stomach, while in rabbits the body of the stomach is most potent.[39]

The metabolism of serotonin is little understood. Apparently 5-hydroxytryptamine can be deaminized by liver tissue since it shares with other amines the ability to increase oxygen uptake by this substrate.[33] On the other hand, evidence has been presented that liver tissue can convert tryptophane to 5-hydroxytryptophane and that this may be decarboxylated by liver and kidney to yield 5-hydroxytryptamine (serotonin).[40, 41] Since serotonin is inactivated by mild oxidants, and since defibrinated blood is devoid of serotonin after perfusion through lung tissue,[35] it has been suggested that enzymatic action is responsible for its destruction.

Although it is clear that serotonin evolution in serum depends upon the presence of platelets, it is not yet apparent whether platelets alone suffice for this result, or whether there must be an interaction of platelets and plasma or serum factors. Such interaction might be of two kinds. Normal coagulation, induced by various plasma factors, may be necessary to assure lysis of platelets and liberation therefrom of serotonin. It is also possible, however, that a plasma factor must interact with a platelet factor to produce serotonin. Earlier in this paper, observations were cited which indicated that mechanical lysis of platelets in plasma by freezing greatly increased the serotonin content of the mixture. Therefore, if a plasma factor is necessary for serotonin evolution, it need not be a factor produced by the coagulation cycle. Judging from the normal serotonin level of the serum of the previously mentioned afibrinogenemic subject, fibrinogen is not essential for serotonin formation. Evidence has been presented by Zucker,[42] however, that platelets yield more serotonin when suspended in recalcified plasma than in saline solutions containing calcium ions and thrombin.

Serotonin, as might be expected from observations of other workers,[10, 43] was reduced in the sera of thrombocytopenic patients. However, it was discovered also to be deficient in a variety of patients with normal or increased platelet numbers and a bleeding tendency. This was true of some, but not true of all patients with hypoprothrombinemia. The hemophilic subjects usually demonstrated a delay in serotonin evolution that paralleled the rate of clotting, but the final amount closely approached the normal. Among the thrombocythemic patients, serotonin was decreased in six of seven instances. None of these patients had normal prothrombin concentrations, yet none was so lacking in this respect as to provide a ready explanation for the deficient serotonin. Failure of platelet lysis because of impaired coagulation or undue platelet stability probably did not explain the lack of serotonin in thrombocythemia because mechanical lysis of these platelets in their own plasma failed to yield normal levels of serotonin. Since the efficacy of the platelets of the thrombocythemic subjects, when transferred to normal plasma, was not investigated, it was not possible to decide whether the lack of a platelet or of a plasma factor was responsible for the diminished serotonin activity.

Except in thrombocytopenic states, diminished serotonin was not associated with impaired platelet function as measured by clot retraction and prothrombin consumption. While both the bleeding time and capillary fragility

were increased in all the thrombocytopenic patients, only the bleeding time was abnormal in the four thrombocythemic patients on whom these tests were performed. Among the hypoprothrombinemic patients with normal platelet numbers, both diminished and normal serotonin concentrations were associated either with prolonged bleeding time, increased capillary fragility, or with no hemostatic defect. Normal serotonin was associated with either increased capillary fragility, or bleeding time, or both in two patients thought to have pseudohemophilia.

Thus the level of serotonin is regularly decreased in thrombocytopenia, and occasionally in certain other hemorrhagic states. Although it is attractive to suppose that the serotonin liberated at the site of a wound contributes to hemostasis through its vasoconstrictor property, there is no simple correlation between serotonin evolution and clinical hemorrhage. Abnormal bleeding, as pointed out by Reid,[43] may occur despite normal serotonin activity, while decreased serotonin need not be associated with hemorrhage. Since the bleeding tendency accompanying thrombocythemia is not well explained by the routine, conventional tests of hemostasis, however, the diminished serotonin activity also associated with this syndrome may, indeed, be partially responsible for the attendant hemorrhagic state.

SUMMARY

Serotonin evolution in the serum of normal individuals and of patients with hemorrhagic disease has been examined employing contraction of bovine artery strips as the indicator. The decreased serotonin in the serum of persons who have thrombocytopenia has been further confirmed, and new observations have been made of decreased serotonin in patients having platelets in normal or increased numbers. Although the hemorrhagic tendencies associated with thrombocytopenia and with thrombocythemia may be partially explained by the diminution of serotonin activity in these states, no direct correlation could be made between serotonin concentration and the several manifestations of abnormal hemostasis in patients with normal platelet numbers.

The author is indebted to Miss Doris O'Connor for technical assistance.

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THE EFFECT OF DRUGS UPON THE COPPER METABOLISM IN HEPATOLENTICULAR DEGENERATION AND IN NORMAL SUBJECTS

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HEPATOLENTICULAR degeneration (Wilson's disease) is associated with certain biochemical abnormalities and disturbances in copper metabolism. Abnormalities that have been reported are a high urinary excretion of copper,[1] an increased absorption of copper through the gut,[2] abnormal aminoaciduria,[3] a low coeruloplasmin level,[4] a marked increase in copper content of both the brain and liver,[5, 6] a low serum copper,[7] and varying degrees of liver dysfunction.[8]

Copper has been the subject of much investigation in recent years because of its importance in body metabolism. There is some relation to erythropoiesis, and in animals copper has some effect on myelinization in the central nervous system. Copper is ingested in the form of food or medication and is absorbed into the intestinal wall. From there it is retained and excreted through the urine and feces. Other routes of excretion are probably through the bile and through active secretion through the intestinal mucosa. In the intestinal tract it is possible that the copper travels some sort of circuit from absorption to excretion. The experiments of Van Ravesteyn[9] on normal patients showed that the excretion of copper in the bile was less regular than in the feces but would rise in the bile after intravenous administration of copper. When copper was given by mouth, about 25 per cent was absorbed through the intestinal wall, and the absorbed copper was gradually eliminated. When copper was injected intravenously, there was an immediate rise in a few hours. Fetal storage of copper in the liver is high, and there is a gradual decrease to the normal adult level between the fifth and fifteenth years. In the circulation the copper is almost equally distributed in the cells and plasma. There are many altered states in which copper is increased in the plasma, cells, or storage organs such as pregnancy, tuberculosis, carcinoma, and cirrhosis. In the plasma, copper is increased in many infections which Cartwright[10] felt may indicate that copper has some relation to defense mechanisms, although the copper increase may only be an evidence of increased metabolic activity of the cells. Hypercupremia in adults has been observed also accompanying and following Hodgkin's disease, acute leukemia, schizophrenia, manic depression, myocardial infarction, and coronary occlusion.[11] Recently Baxter and Van Wyk[12] produced a disease consisting of a diffuse osteoporosis in young dogs made severely deficient in copper. These same authors[13] reported an anemia

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in dogs as a result of copper deficiency which was characterized by a reduction in the number of erythrocytes with the maintenance of relatively normal red cell indices.

Serum copper is almost entirely in the form of a metalloprotein complex, no detectable diffusible copper being obtained on dialysis through collodion membranes. Holmberg and Laurell[14] claimed that most, if not all, serum copper is contained in a single pure protein which they have prepared from pig serum and from human pregnancy serum. Coeruloplasmin was found to be a blue-colored globulin of molecular weight about 150,000, each molecule containing eight atoms of copper. On electrophoretic analysis it was shown to be an alpha-globulin. Scheinberg and Gitlin[4] have produced evidence that coeruloplasmin is deficient in Wilson's disease. They have suggested that Wilson's disease is an example of a disease caused by a deficiency of a specific plasma protein. Investigations by Uzman and Denny-Brown[3] have shown excessive urinary amino acid excretion to be a very consistent feature of hepatolenticular degeneration even in early and mild states of the disease. In all three of the cases presented here, an elevated urinary amino acid excretion was present.

The normal range of serum copper has been established to be between 86 and 161 mg. per 100 ml.[15] Estimations of normal subjects have all given values within this range. Estimations of urine copper excretion per twenty-four hours have given values ranging from 0 to 69 μg.[16] In three cases of Wilson's disease studied by us, the serum copper levels were 61.6, 77.4, and 136.4 μg per 100 ml. The twenty-four hour urinary excretions for the same patients were 197.8, 556, and 378 μg, respectively. Hypocupremia has been noted only in hepatolenticular degeneration and in one patient with severe nephritis and hypoalbuminemia.[17]

The present study is undertaken to determine the effects of various drugs upon the copper metabolism in both normal subjects and in patients with Wilson's disease. All the three patients are from a single family and are suffering from the more chronic "pseudosclerotic" type of hepatolenticular degeneration, with a history of tremor in the upper limbs and dysarthria progressing to titubation of the head and trunk, and dystonic rigidity. The age of onset has varied from 17 to 29 years, and the illness has run a course of five, fourteen, and fifteen years. One other sibling died in 1947 from hepatolenticular degeneration after an illness of twenty years.

Electrophoretic studies of the plasma protein in our cases have been normal. Another interesting observation has been the consistently low serum uric acid level. The highest level found in our patients with Wilson's disease has been 1.75 mg. per cent. Preliminary studies have shown the uric acid pool size to be reduced and the turnover rate to be very high. These studies are being pursued further.

METHODS

Each patient was given a thorough history and physical examination and then placed on a complete balance study. Analysis of the diet for copper was carried out. The copper in-

take was measured by taking an aliquot of the food and liquid consumed for each three-day period. Urinalyses were performed on three-day pools; serum copper was determined weekly; and fecal copper was analyzed with three-day specimens. Each patient was placed on a six-day control period and then given the drugs for the prescribed treatment period. Copper was estimated essentially in the urine, feces, and food, using a modification of the method of A. Eden and H. H. Green.[18] Determination of the copper in the serum was based on the diethldithiocarbamate reaction described by Gubler and associates.[19] Special care was taken to see that all receptacles for the urine, all syringes for the collection of blood, and all apparatus used were copper free. Control blanks were put up each time. In addition to routine laboratory tests, other tests included fasting blood sugar, serum bilirubin, Bromsulphalein, nonprotein nitrogen, serum sodium and potassium, alkaline phosphatase, and serum calcium.

RESULTS

BAL.—In our previous studies with hepatolenticular degeneration, we found a reduced excretion of copper via the stools.[2] Although there was a much greater excretion of copper in the urine, as compared to the normal, patients with Wilson's disease were in a marked positive copper balance.

The substance 2, 3-dimercaptopropanol (British Anti-Lewisite, BAL) was originally introduced by Peters and his associates[20, 21] as an antidote for the effects of arsenic gases. Mandelbrote and his associates[1] reported a high urinary copper excretion in a case of Wilson's disease and found that in this condition, as in normal persons, the administration of BAL greatly increased copper excretion in the urine. We have found similar results along with Cummings,[22] Denny-Brown,[23] and others. Each one of our three patients was given BAL in a dosage schedule suggested by Cummings.[22] Evidences of toxic reactions such as transient nausea, dizziness, occasional vomiting, and blurring of vision, commonly associated with the administration of BAL, were encountered. In one of our cases, hematemesis and blurring of vision occurred. It is important to insure that injections are deeply intramuscular and are not made near nerves, as subcutaneous injections cause painful lumps. Improvement after a course of BAL usually begins from ten to fourteen days after the course and continues during the next two or three months. Hepatic insufficiency in our patients already present showed no change that was considered significant after multiple exposures to BAL. The administration of BAL not only increased the excretion of copper from the daily intake, but also from copper which had been stored in the tissues of the body. The patient in Case 1 showed a fivefold increase in the urinary excretion of copper, and the patients in Cases 2 and 3 showed a twofold and fourfold increase, respectively. All of the patients showed some clinical improvement. This was indicated by an increase in the amount of daily living tasks the patients could perform, the graying of the Kayser-Fleischer rings, and changes noted in the neurological examination.

Fig. 1 shows the results of a balance study in a patient, V. L., with Wilson's disease. In the first two periods, there is a marked excretion of copper in the urine and a positive balance. During the administration of BAL, there is a further increase in urinary copper excretion. At this time the patient is in a negative balance. The percentage of copper that appears in the

stool as compared to the intake varies from 30 to 78 per cent with a mean of 41.5 per cent. A normal subject, M. D., shows a marked contrast with over 90 per cent of the copper intake being received in the stool and a normal amount excreted in the urine. When both patients are given an increased intake of copper orally, the normal subject excretes it in the stool, while the

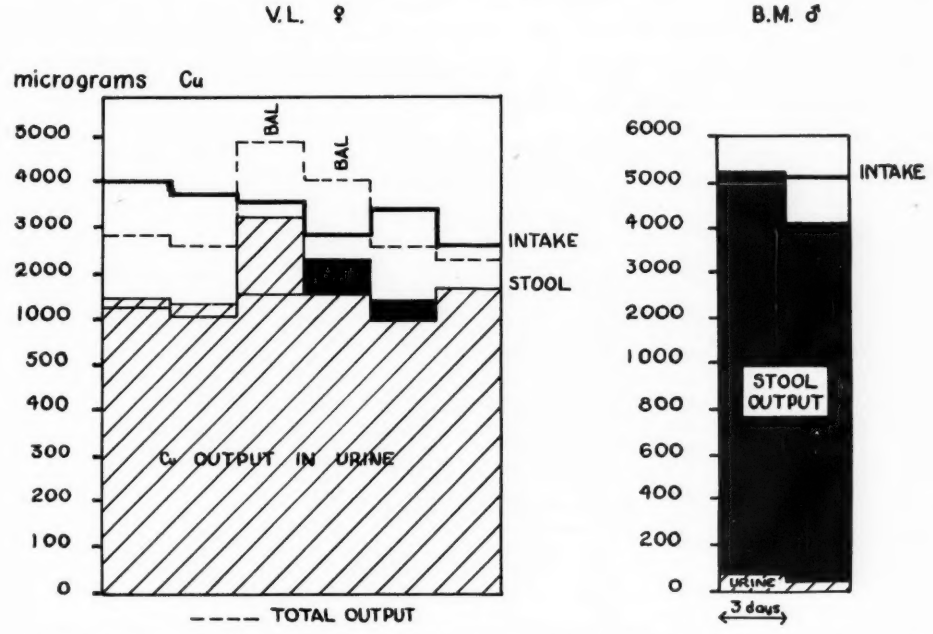

Fig. 1.—Shows a balance study on V. L. with Wilson's disease, and a normal control, B. M. In the first two periods, V. L. shows a positive balance of copper. The dotted line represents the total output of copper. On BAL, a negative balance of copper is present. When BAL was discontinued in the last two periods, a positive balance was again present. The marked contrast in the amount excreted and the amount of copper appearing in the stool can be seen in the normal subject.

patient with Wilson's disease absorbs most of it and increases copper retention.

Fig. 2 shows the results expressed in percentage of copper in a patient, I. P., with Wilson's disease and a normal control.

Ion Exchange Resins.—Because exchange resins have been used to remove the sodium in patients with heart failure, we were interested to see if this material would remove copper and possibly give us another method of treatment for patients with Wilson's disease. After a control period, the patients were placed on 15 Gm. of sodium polystyrene sulphonate resin three times daily.* Fig. 3 shows the effect on two patients. There is a definite increase in the stool copper. On a normal diet, a negative balance of copper was obtained in both patients, W. P. with Wilson's disease, and M. D., a normal subject. When Patient W. P. was given 3,000 mg. of copper orally, in addition to his regular diet, a negative balance was not obtained. This would tend to emphasize the fact that these patients should be kept on the lowest copper diet possible.

*Supplied by Dr. Evan W. McChesney of Sterling-Winthrop Research Institute, Rensselaer, N. Y.

Studies with an ion exchange resin, Carbo-resin,* have shown the same effect on copper. With the resin and a regular diet, W. P. continued to show definite clinical improvement. This clinical improvement has reached its highest peak in the three years that we have observed him.

In continued balance studies it was found that the copper became refractory to absorption by the resin. In omitting the resin for periods of one to two weeks, however, a negative balance was again obtained. We are now using an interrupted treatment schedule with resin for one month, then off for two weeks, and again on for one month.

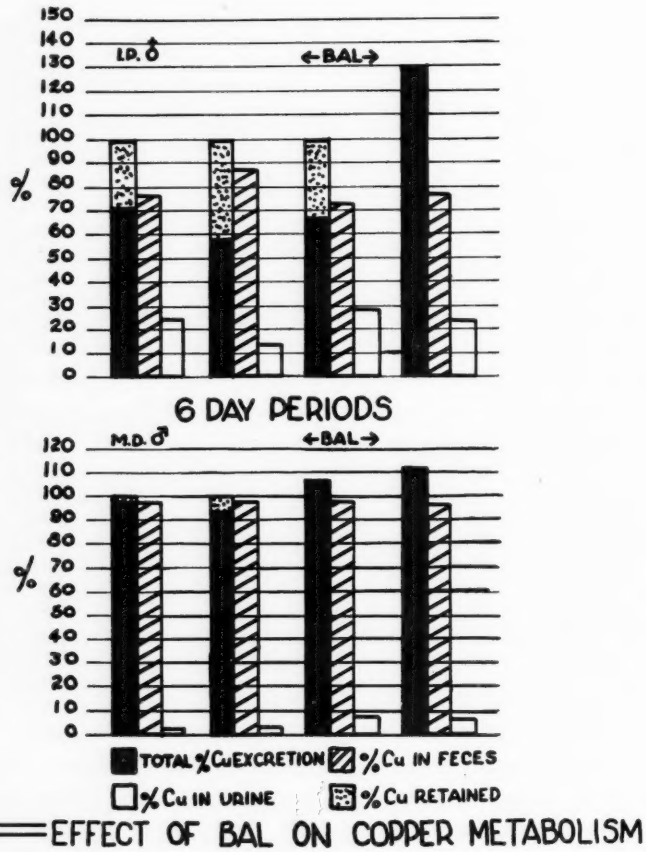

Fig. 2.—Shows sixday periods during balance study in Patient I. P., with Wilson's disease and a normal control, M. D. The stippled area represents retained copper expressed in per cent. Values above 100 per cent represent a negative balance. In Patient I. P., a lag period is present before BAL produces a negative balance. The normal control reacts to BAL, also showing a negative balance of copper with no lag period.

The resin also appeared to influence the excretion of amino acids. A marked increase in the twenty-four hour urinary excretion appeared during the resin therapy which did not parallel the urinary copper excretion. This aspect is being investigated further.

*Carbo-resin, ion exchange resin supplied by Eli Lilly and Company, Indianapolis, Ind.

Adrenocorticotrophic Hormone.—The effect of ACTH in a patient with hepatolenticular degeneration and in a normal subject was observed. The patient, W. P., with Wilson's disease, after a normal control period, was given 20 units of ACTH intramuscularly daily for six days. During the first three-day period of this medication, there was a marked negative balance of copper. In the second three-day period of the medication, however, the patient returned to a positive balance. This experiment has not been repeated in this patient because of the marked increase in symptoms following this medication. His tremor became much worse, and his general appearance deteriorated. In

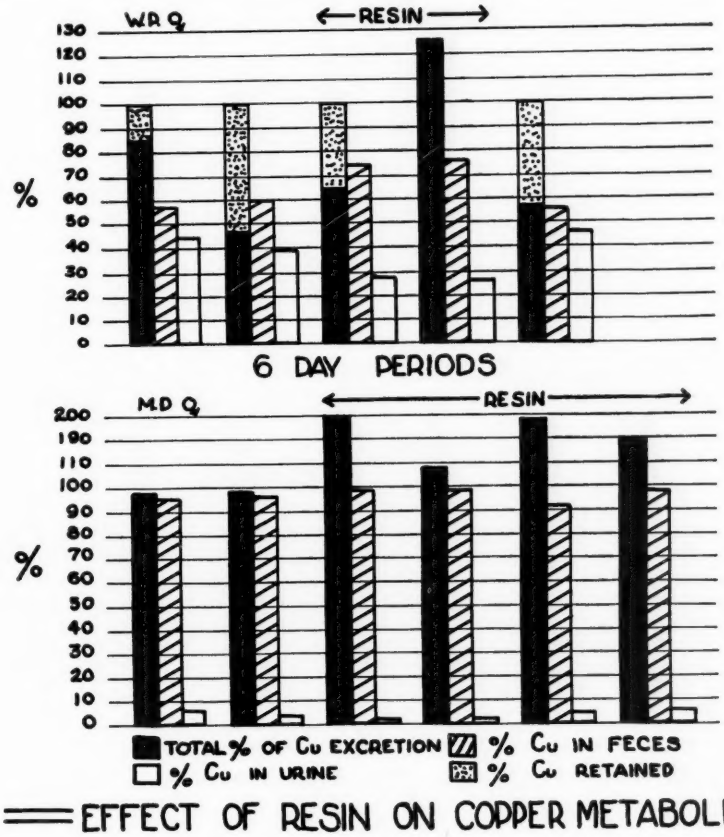

Fig. 3.—Continuous balance study in a patient with Wilson's disease, W. P., and a normal subject, M. D. Black bars represent percentage of total copper intake that is excreted. Stippled bars represent copper retention. W. P. shows a negative balance of copper while on resin. There is a slight lag period before this occurs, and a positive balance occurs when resin is discontinued. M. D. was continued on resin for a longer period and continues to show a negative balance throughout.

the normal control subject, ACTH showed no appreciable effect upon the copper balance studies. This is shown in Fig. 4. The exact significance of this observation is not entirely clear at this point. The experiment has not been repeated for reasons stated previously. Whether ACTH has some indirect or direct effect upon the small bowel may be postulated, but the results of this one experiment are not conclusive.

Lahey and his associates[17] have reported that in patients with acute leukemia the administration of the adrenocorticotrophic hormone (ACTH) was accompanied by a decrease in blood copper values. This occurred whether or not a clinical remission was induced by such therapy. We did not follow serum copper levels closely enough to determine what occurred in our patient, but the total copper excretion definitely increased.

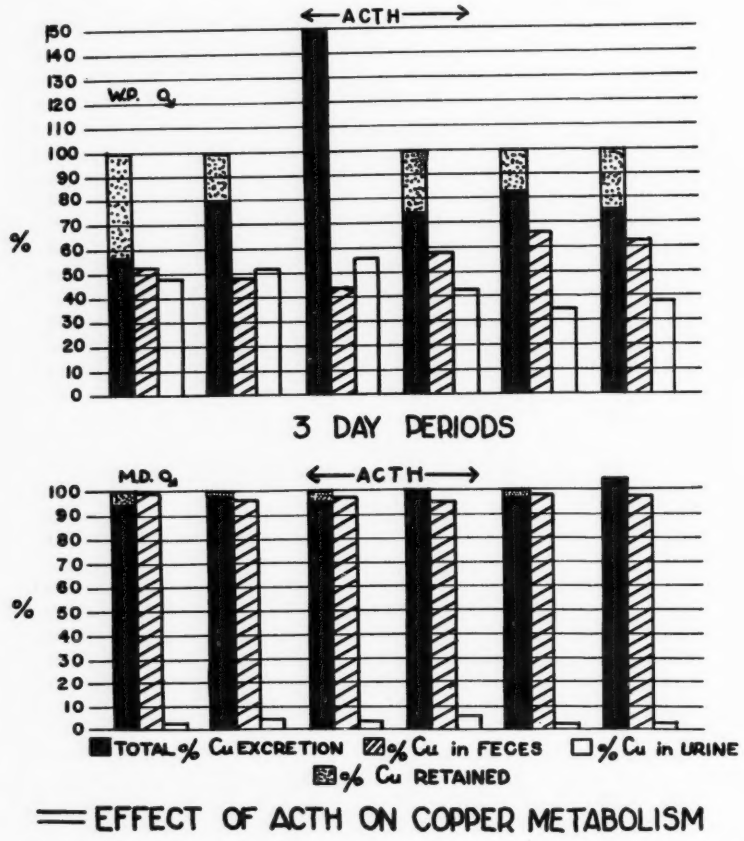

Fig. 4.—Shows the effect of ACTH upon the copper balance in Patient W. P., and a normal subject, M. D. ACTH caused a marked excretion in the total copper, while it had no appreciable effect in the normal control.

Effect of a Chelating Agent.—Recently an organic chelating agent, Versene,* disodium calcium ethylene diamine tetra-acetate (CaEDTA)* has been suggested for the treatment of lead poisoning.[24] Versene is stable, odorless, water soluble, and forms a stable complex with lead which is virtually non-ionized. It is not concentrated in any particular organs or tissues of the body, is readily excreted in the urine, and apparently has little or no toxicity for man in the dosages used. Because of the effect upon urinary lead excretion, this drug was tried on patients with Wilson's disease.

The oral daily dose used in adults was 30 mg. per kilogram. For study purposes, a course of five days of continuous therapy was interrupted for a

*Supplied in ampules and as tablets by Riker Laboratories, Los Angeles, Calif.

period of five days, and then a repeat course of five days was given. In the second five-day period the daily dose was increased to 60 mg. per kilogram. The serum levels of calcium, phosphorus, sodium, potassium, and nonprotein nitrogen were followed. Multiple hemograms and urinalyses were made. The intravenous dosage schedule used for adults was 2 Gm. a day for a period of five days. One milliliter from an ampule containing 1 Gm. calcium disodium versenate in 5 ml. was diluted with 250 ml. of 5 per cent glucose and admin-

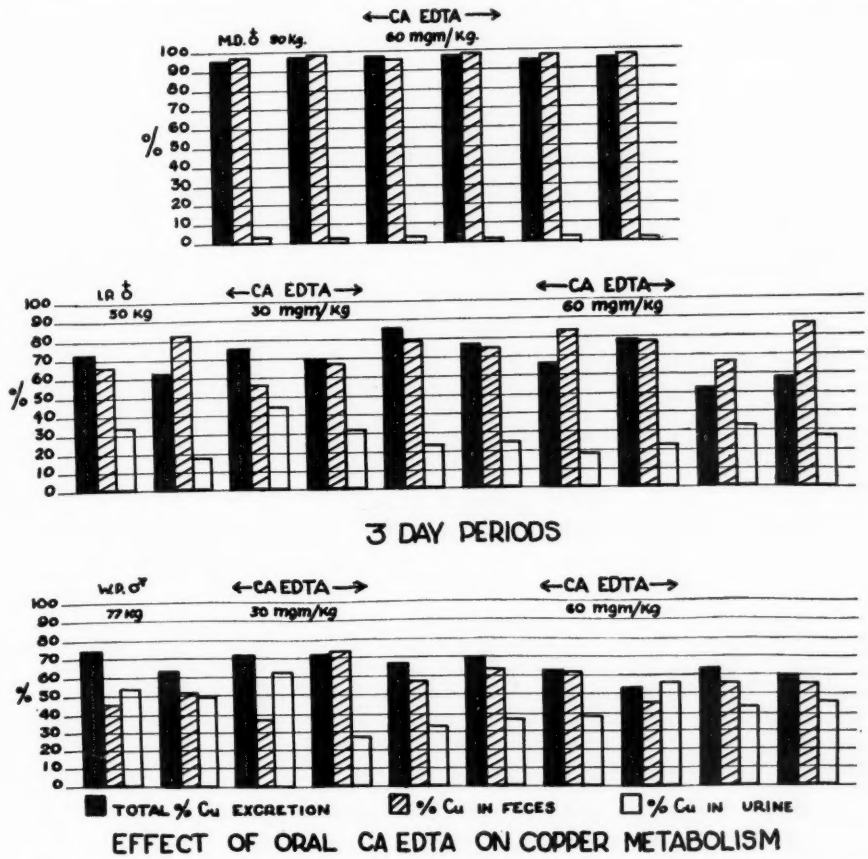

Fig. 5.—Shows the effect of Versene in Patients I. P. and W. P., and in a normal subject, M. D. In I. P., there was a slight increase in the total excretion of copper with a dose of 30 mg. per kilogram. This appeared after the drug was stopped. With an increased dose of 60 mg. per kilogram, a slight increase was again noticed. In Patients W. P. and M. D., no appreciable increase in total copper excretion was detected.

istered intravenously over a period of one hour. If no untoward reactions were observed after an interval of no less than four hours, 1 Gm. was given in 500 ml. of isotonic glucose administered over one hour. This dose was administered twice daily for a period of five days, followed by two days' rest and five more days of therapy. No untoward signs were observed either clinically or in laboratory findings during the oral or intravenous dosage schedule. Fig. 5 shows the results of the oral dosage. In *I. P.* there is a slight increase in the total excretion of copper. A lag period was present, and the increase occurred only after the five-day course had stopped. In the second

period 60 mg. per kilogram was given. A slight increase in copper excretion occurred during the second three-day period. In Patient W. P., during both dosage schedules, there was no appreciable effect upon total excretion of copper. In Patient M. D., normal control subject, on a dosage schedule of 60 mg. per kilogram, there is no change in the total excretion of copper or in the percentage of copper in the urine. Fig. 6 shows the effect of the intravenous dosage. In both patients, W. P. and I. P., with Wilson's disease, there is an increase in the total excretion of copper during both periods. In Patient I. P. the increase was more marked during her second course of intravenous therapy.

DISCUSSION

A high serum copper in Wilson's disease was first reported by Glazebrook[6] and in two cases reported by Cummings.[22] Normal values were ob-

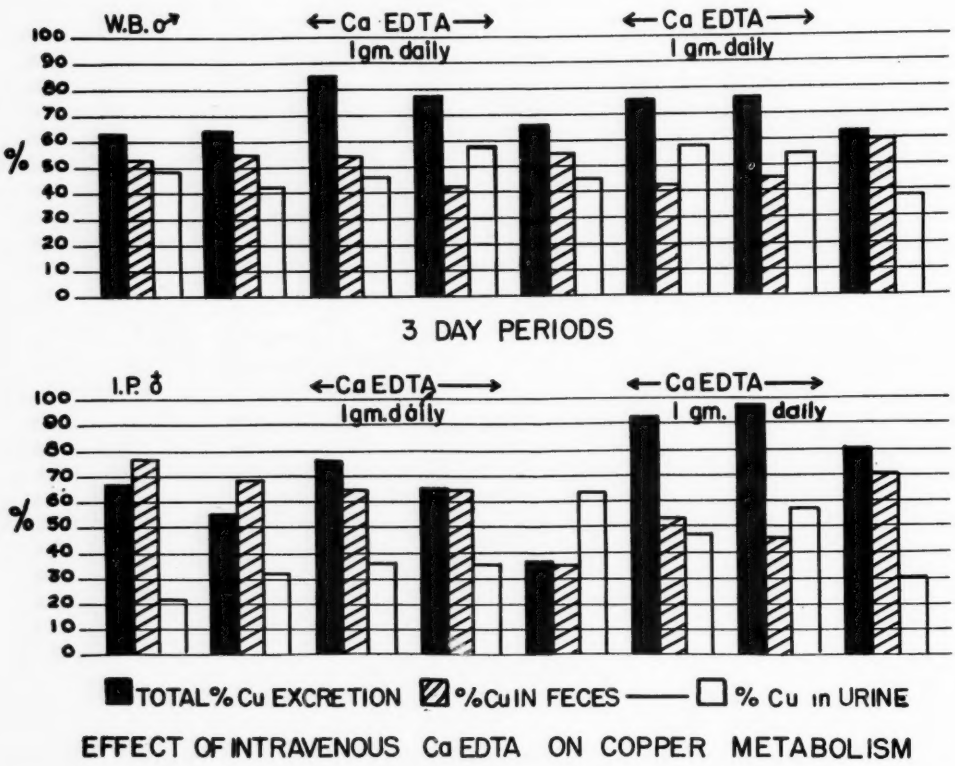

Fig. 6.—Intravenous Versene (CaEDTA) produced an increase in the total copper excretion in two patients with Wilson's disease. This was more marked in Patient I. P. However, neither the oral nor the intravenous administration of this drug in the doses used produced a negative balance of copper.

tained in two cases reported by Matthews[16] and in one reported by Brinton.[25] Although Denny-Brown and Porter[23] did not report observations on serum copper, they suggested at the time that the serum copper was probably raised and overflowed into the urine due to a low renal threshold. In two of our three cases the serum copper was low, and in the third it was within normal

limits. Bearn[7] found a serum copper lower than normal in fifteen of seventeen cases. In the two remaining, the serum copper was found to be in the low normal range.

The presence of a low serum copper and high urinary excretion of copper is of diagnostic importance in Wilson's disease. When this is accompanied by aminoaciduria, a low coeruloplasmin level, and increased absorption of copper through the gut, one may be able to establish a diagnosis of Wilson's disease even though symptoms may be minimal. The excess copper that is absorbed is deposited in the tissues, probably mostly in the liver and brain. With the administration of such agents as BAL and Versene, tissue copper is excreted and appears in the urine in large amounts. The improvement following these agents is noticed in the neurological signs and symptoms and suggests that this copper is probably from brain depots. When ion exchange resins are given, the excess absorption of copper through the gut is markedly reduced. A combined treatment schedule of both BAL and Versene and resin would probably benefit a patient with Wilson's disease. The removal of excessive amounts of tissue copper with BAL, followed by a continued reduction of copper absorption over a long period of time, has caused marked clinical improvement in our patients.

SUMMARY

The effect of BAL (2, 3-dimercaptopropanol), Versene (disodium calcium ethylene diamine tetra-acetate), ion exchange resins, and adrenocorticotrophic hormone (ACTH) on the copper metabolism in patients with Wilson's disease and in normal subjects has been studied. Because of a marked increase of copper absorption through the gut in Wilson's disease, it is recommended that these patients be placed on a low copper diet plus interrupted resin therapy between their periods of treatment with BAL or Versene.

The technical assistance of Miss Vera Rosenberg is gratefully acknowledged.

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PAPER ELECTROPHORETIC ESTIMATION OF PROTEINS IN CEREBROSPINAL FLUID

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MANY diseases of the nervous system involve changes in the protein composition of the cerebrospinal fluid. It would therefore be of value to have a simple method by which the distribution pattern of the proteins in pathologic fluids can be studied and correlated with the disease. Paper electrophoresis is a technique that is eminently suitable for this purpose. It requires a relatively small quantity of fluid and gives a clear-cut separation of the proteins into individual components of albumin, alpha-1, alpha-2, beta and gamma globulins. The classical electrophoresis of moving boundaries developed by Tiselius provided the foundation for this method that was developed concurrently in this country by Durrum[1] and in Sweden by Cremer and Tiselius.[2] Schneider and Wallenius[3] applied the method to cerebrospinal fluid. The present paper is concerned with a simplified method for the application of paper electrophoresis for the quantitative determination of the proteins in cerebrospinal fluid.

EXPERIMENTAL

Concentration of the Fluid.—Since the cerebrospinal fluid has a very low protein content, it cannot be used directly for paper electrophoretic measurement, but must be concentrated twenty-five- to a hundredfold, depending upon the protein content. Since the proteins are easily denatured, this becomes a rather difficult task. Various methods were tried including dialysis against high molecular weight colloids such as dextran[3, 4] and polyvinyl pyrrolidine,[5] perevaporation and lyophilization after the removal of salts by ion exchange treatment. The latter method was recently reported[6] to give satisfactory results.

The best results can be obtained by precipitation of the proteins with ice-cold acetone, a simplification of the method of Bucher and associates.[7] About 10 ml. of cerebrospinal fluid are centrifuged to remove cells in cold cups, or, if available, in a refrigerated centrifuge. The clear fluid is transferred to a 40 ml. Pyrex centrifuge tube and precipitated with double its volume of cold acetone. After careful mixing, the tube is covered immediately with Parafilm and placed in the freezer for about twelve hours, usually overnight. The mixture is centrifuged cold, the supernatant solution poured off, and the centrifuge tube left open in the refrigerator to remove traces of acetone. A small quantity of Versene* (5 to 10 mg.) is added with a microspatula; it was found that Versene is more effective when added as a solid than in solution. Into this mixture 0.1 to 0.25 ml. of 0.9 per cent sodium chloride solution is pipetted and stirred vigorously to bring the proteins into solution. Barbital buffer pH 8.6 can also be used for dissolving the proteins.

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*Disodium salt of ethylenediamine tetra-acetic acid. (Bersworth Chemical Co., Framingham, Mass.)

Paper Electrophoresis.—For the paper electrophoretic procedure, the same experimental arrangement was used as reported by Durrum,[1] using barbital buffer of pH 8.6 and a potential of 240 volts applied to the electrodes for six hours. After this time, the paper strips are carefully removed and the protein localized on the paper by denaturation through drying at 80° C. for fifteen minutes. Then the strips are left in bromphenol blue solution overnight. For the removal of excess dye, 0.5 to 1.5 per cent acetic acid is recommended followed by washing with a solution containing 1.5 per cent each of sodium acetate and acetic acid.[8] The strips are then dried at 60° C.

Another treatment for removing the dye was reported by Cremer and Tiselius,[2] using mercuric chloride in methyl alcohol followed by mercuric chloride in ethyl alcohol, then washing with methyl alcohol and finally ether. It was found that by the use of these organic solvents a clearer picture could be obtained, the individual stained proteins emerging from a nearly white background, which is not the case when acetic acid is used. Since, however, too much of the color is removed by the organic solvent washing, this method of washing is recommended for blood serum rather than cerebrospinal fluid, with the exception of some pathologic fluids in which the protein content is high. We obtained good results, however, by combining the two methods, first fixing the color with 0.5 per cent acetic acid, then applying the mercuric chloride method. If, by incorrect washing, the color becomes too pale, it is possible to redye the proteins by again placing the paper in the dye bath.

The paper strips are read in an electronic densitometer.* For measurement with this apparatus, it is not necessary that the paper be made translucent by immersion in oil. The instrument consists of a sensitive electronic photometer which measures the light passing through the dyed paper strip at any point, a search unit, and an amplifier. Direct plotting of the density curves is possible. We found it advantageous, however, to record the successive density readings and plot the curve afterward on a larger scale. The electrophoretic diagrams can be evaluated by the use of a planimeter, the diagram being subdivided into areas representing the protein fractions by dropping perpendiculars from the minima on the curve to the base line.

Conversion Factor for Gamma Globulin.—Quantitative evaluation of the measured surface is based on the assumption that the bound dye on the paper strips is directly proportional to the concentration of the separated protein fractions. Several authors reported that there is a difference between the affinities of gamma globulin and albumin for the dye bromphenol blue, and that a factor is needed to express values for gamma globulin. We investigated the color absorption of highly purified blood proteins† under the same experimental conditions used in the paper electrophoresis of blood serum and cerebrospinal fluid.

Based upon normal serum with a total protein of 7,200 mg. per 100 ml. and 56 per cent albumin and 16 per cent gamma globulin, 202 mg. albumin and 57 mg. gamma globulin were dissolved in 5 ml. physiologic saline solution. The ratio between albumin and gamma globulin is 3.54 in this mixture.

RESULTS

The paper electrophoretic measurements were carried out in four replications as described for cerebrospinal fluid. The albumin and gamma globulin moved on the paper the same distance as in fresh serum or cerebrospinal fluid, a proof that these proteins were not denatured. After staining, the separated gamma globulin and albumin were measured in the densitometer and, by use of the planimeter, the ratio of albumin to gamma globulin calculated. An average value of 3.67 was obtained. Since the theoretical value is 3.54, it is

*Model 525 made by the Photovolt Corporation, 95 Madison Ave., New York, N. Y.

†We wish to express our thanks to Dr. D. M. Surgenor of Harvard University, who kindly supplied these proteins.

obvious that albumin and gamma globulin combine with bromphenol blue to an almost equal extent, and it is unnecessary to use a special conversion factor for gamma globulin.

Concentration Experiment.—In order to ascertain that the process of concentration does not affect the electrophoretic distribution of the proteins, experiments were carried out as follows:

a. Albumin and gamma globulin in saline solution: 0.5 ml. physiologic saline solution containing 20.2 mg. albumin and 5.7 mg. gamma globulin was diluted to 10 ml. and precipitated with 20 ml. acetone, as described for cerebrospinal fluid. The precipitate was redissolved in 0.5 ml. physiologic saline with the addition of Versene, and paper electrophoretic analysis was carried out. The diagrams were evaluated quantitatively and the ratio of albumin to gamma globulin was calculated, an average value of 3.53 being obtained in comparison with the ratio of 3.64 for the solution without precipitation.

b. Diluted blood serum: 0.2 ml. blood serum was diluted to 20 ml. with physiologic saline solution and precipitated with 15 ml. cold acetone. The precipitate was redissolved in 0.4 ml. of saline solution with the addition of Versene as described for cerebrospinal fluid. The numerical values for the serum proteins before and after concentration are presented in Table I. The results show conclusively that the acetone treatment does not change the protein pattern.

TABLE I. THE INFLUENCE OF ACETONE PRECIPITATION ON THE RELATIVE COMPOSITION OF SERUM PROTEINS*

		GLOBULINS			
	ALBUMIN PER CENT	ALPHA-1 PER CENT	ALPHA-2 PER CENT	BETA PER CENT	GAMMA PER CENT
Before precipitation	50.7	5.9	8.7	14.4	20.2
After precipitation	50.6	4.6	8.8	15.2	21.0

*Pathologic serum.

DISCUSSION

A few comments should be made on handling and analyzing the fluid after the spinal tap. If the fluid cannot be worked up immediately, it should be placed in the refrigerator. A temperature close to freezing point should be maintained in every step of the procedure. Proteins of the cerebrospinal fluid denature easily; denaturation is recognizable after staining in the form of a broad line on the paper at the position of the apex where the sample is placed. If proper conditions are observed, the acetone itself does not affect the migration of the proteins as was shown by two experiments, one with a mixture of albumin and globulin and one with blood serum.

Paper electrophoresis may be used both qualitatively and quantitatively.

For the qualitative interpretation, the shape of the curve is the determining factor to suggest abnormality in a fluid. In most cases this is obvious because the pattern of the pathologic fluid is strikingly different from the normal. Although some investigators[5] believe that the visual impression of the

shape of the pattern is adequate for ordinary diagnostic purposes, and do not present the relative percentages of the various protein components, we feel that both presentations are important, with more emphasis on the quantitative distribution. Probably what has most hampered the general acceptance of quantitative presentation is that there is no good agreement between various authors on the evaluation of the diagrams. Paper electrophoresis is based in principle on the classical Tiselius type of electrophoresis. Since this is a well-established technique, it seemed obvious to use this technique as a basis for comparison of quantitative results. Since the values for gamma globulin were found to be lower by paper electrophoresis, adjustments were made by the use of a special factor by which gamma globulin values were to be multiplied. Koiw and co-workers[9] recommended a conversion factor of 1.4,[10] they analyzed various pathologic fluids by both methods, found great variation in the factor, ranging from 1.1 to 2.1, depending upon the disease. Cremer and Tiselius[2] recommended a factor of 1.6, but later Kunkel and Tiselius[8] found that a factor of 1.3 is sufficient. Different methods of washing the papers may be an influence in the variability of this factor.

Most of these authors obtained their diagrams by cutting up the strips every 5 mm., necessitating some 40 to 50 extractions and colorimetric measurements. Instability of the color in alkaline solution during the lengthy procedure might itself be a source of error.

TABLE II.—THE RELATIVE COMPOSITION OF CEREBROSPINAL FLUID AND BLOOD SERUM IN NORMAL AND SOME PATHOLOGIC CONDITIONS

		CEREBROSPINAL FLUID PROTEIN					BLOOD SERUM PROTEIN				
		ALBUMIN	GLOBULINS				ALBUMIN	GLOBULINS			
PATIENT NUMBER	DIAGNOSIS	PER CENT	α-1 PER CENT	α-2 PER CENT	β PER CENT	γ PER CENT	PER CENT	α-1 PER CENT	α-2 PER CENT	β PER CENT	γ PER CENT
216 (I)*	Normal	57.3	6.1	11.0	9.2	16.5	63.9	2.8	8.2	9.6	16.7
224 (II)	Brain tumor	45.6	2.1	3.2	28.4	20.6	48.2	2.6	4.7	14.1	30.4
103 (III)	Guillain-Barré	52.8	4.8	9.6	24.1	8.6	40.1	9.9	11.6	16.2	21.3
159 (IV)	Multiple myeloma†	34.5	10.3	6.9	21.2	26.9	43.8	4.6	13.4	5.7	41.2

*Numbers in parentheses refer to Fig. 1.
†With neurological disorder.

We found that by directly measuring the stained paper with a precision electronic densitometer there is no difference in color absorption between albumin and globulin. Although this is contradictory to the findings of the afore-mentioned investigators, it is in good agreement with Pezald and co-workers[11] who determined the binding of denatured serum albumin and gamma globulin, using 1 mg. of the protein. They found that although from azocarmine 0.20 mg. is taken up by serum albumin and only 0.11 mg. by gamma globulin, and from amido black 0.31 mg. by albumin and 0.2 mg. by globulin, from bromphenol blue the quantities were nearly the same; namely, 0.13 mg. by albumin and 0.12 mg. by gamma globulin. Since the affinity of albumin and gamma globulin for bromphenol blue was found to be practically identical by

these authors in terms of absorption, and by us under the conditions of actual electrophoretic measurement, it is apparent that there is no need for a correction factor for gamma globulin.

Great numbers of cerebrospinal fluid samples from patients with various neurological disorders have already been studied and quantitatively evaluated. Since we found that in some cases the protein composition of the blood changes, we are now examining not only the cerebrospinal fluid, but also the blood. We found, interestingly enough, that the blood sometimes shows a different picture from the fluid. To illustrate this point, we selected a few diagrams of pathologic fluids and sera in comparison with the normal. (Fig. 1 and Table II.)

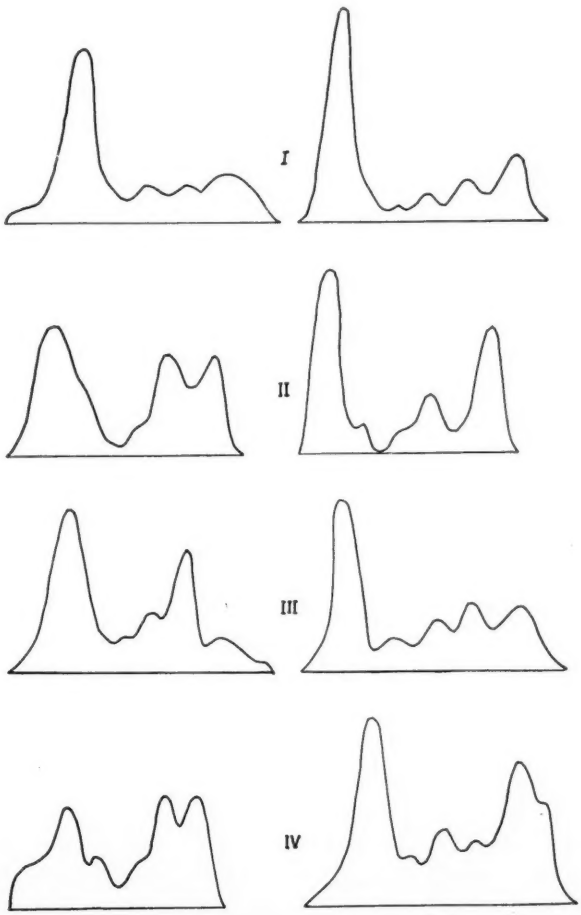

Fig. 1.—Paper electrophroretic diagrams of the protein composition of cerebrospinal fluid (left) and blood serum (right). I, Normal; II, brain tumor; III, Guillain-Barré; IV, multiple myeloma with neurological disorder.

The application of paper electrophoresis by many investigators in neurochemistry will bring profound changes in our knowledge of the protein chemistry of pathologic cerebrospinal fluid and blood. With this new and relatively

simple technique, studies can be extended to many diseases. The information obtained in the course of these investigations not only will be useful in the diagnosis of the various neurological diseases, but will contribute to the basic knowledge of blood and cerebrospinal fluid.

SUMMARY

A simplified method of concentrating cerebrospinal fluid is presented. The method of concentration was tested by using a mixture of albumin and globulin, and a blood serum diluted 100 times. The procedure for quantitative paper electrophoresis of the resulting solution is described.

The conversion factor recommended for gamma globulin was reinvestigated. It was found that under the experimental conditions applied, taking readings with an electronic densitometer, the absorption of the dye bromphenol blue by albumin and globulin is the same. Therefore, a conversion factor is unnecessary.

The method is applicable to clinical laboratory use.

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NORMAL HUMAN OXYGEN SATURATION OF THE ARTERIAL BLOOD AS DETERMINED BY GASOMETRIC ANALYSIS

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CORRECT methods for determination of normal human arterial oxygen saturation are of basic research and clinical importance. The techniques employed to make this measurement are photometric, spectrometric, and gasometric. The photometric and spectrometric methods are based on the differential spectral characteristics of oxyhemoglobin and reduced hemoglobin.[1] The gasometric procedure for measuring the percentage of oxygen saturation of the hemoglobin is done with the Van Slyke manometric apparatus.[2] The oxygen content and capacity of the arterial blood sample obtained by the latter method are used to calculate the percentage oxygen saturation of the hemoglobin. The formula for making this calculation is:

$$\text{Per cent oxygen saturation of the hemoglobin} = \frac{\text{Oxygen content of the hemoglobin Vols. \%} - \text{Dissolved oxygen Vols. \%}}{\text{Oxygen capacity of the hemoglobin Vols. \%} - \text{Dissolved oxygen Vols. \%}}$$

During the last fifteen years, a wide range of normal values for arterial oxygen saturation has been reported in the literature. Keys and Sneil,[3] in 1938, summarizing gasometric determinations done by other workers, reported an average normal saturation of 95.0 per cent, with a range of 93.0 to 98.0 per cent. Six years later, Roughton, Darling, and Root[4] stated that the normal value should be 97 per cent and conducted experiments to show that the oxygen capacity of arterial blood, when determined by saturation with air in a rotating tonometer, was 2 per cent higher than the value of the arterial blood in vivo. Drabkin and Schmidt,[5] by means of a spectophotometric technique, found a mean value for six normal men of 98.6 per cent with a range of 98.0 to 99.3 per cent. Comroe and Walker,[6] in 1948, using an in vivo equilibration technique, obtained a mean oxygen saturation of 97.5 per cent with a range of 93.6 to 101.0 per cent; using an in vitro technique a mean of 97.4 per cent with a range of 93.2 to 101.4 per cent; and with the oximeter a mean of 96.2 per cent with a range of 94.0 to 98.0 per cent. In calibrating a photoelectric oximeter by Van Slyke analyses, Wood and Geraci[7, 8] found a normal arterial oxygen saturation of 97.9 per cent with a range of 94.1 to 101.0 per cent.

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Photometric and spectrometric equipment for measuring the percentage of oxygen saturation of the blood must be standardized with the gasometric apparatus. It is the purpose of this study to compare gasometric methods of determining blood oxygen content and capacity, and to find the correct value for normal arterial oxygen saturation.

MATERIAL AND METHODS

General.—Arterial blood samples were drawn into syringes with oiled plungers. A small quantity of heparin was drawn into each syringe and the air expelled, leaving 0.1 ml. of heparin to fill the tip of the syringe. Samples were taken from an indwelling arterial needle. A small amount of chemically clean mercury was drawn into the syringes to facilitate mixing. They were capped and no air bubbles were visible in any of the samples. The syringes were immediately placed in ice water and kept in the dark.

Gasometric Techniques.—Analyses were carried out in duplicate at 839 feet above sea level. The mean barometric pressure was 738 mm. Hg. Duplicate determinations agreed within 0.2 volume per cent or less.

Oxygen contents were measured by the neutral ferricyanide method of Van Slyke and Neill[2] and by the alkaline ferricyanide method of Van Slyke.[9] The neutral ferricyanide solution contained 6 Gm. of potassium ferricyanide and 10 Gm. of saponin* made up to 1 liter with distilled water. Each lot of saponin was tested for hemolytic activity. Ten grams of saponin were made up to 1 liter with normal saline solution. Seven-tenths milliliter of this solution was placed in a test tube and 0.2 ml. of fresh blood added. The tube was shaken and the time required for hemolysis was noted. The saponin used caused hemolysis within a period of five to six seconds.

Normal arterial blood was considered to contain 0.3 volume per cent of physically dissolved oxygen[10, 11] which was subtracted from each oxygen content.

Oxygen capacities were measured by the neutral or alkaline ferricyanide-tonometer techniques. Ten milliliters of blood were rotated at 38° C. for fifteen minutes in a 200 ml. separatory funnel (tonometer) open to the air. One milliliter of blood, without removing the tonometer from the constant temperature water bath, was quickly transferred by pipette and analyzed by the neutral or alkaline ferricyanide content method. The temperature of the blood during rotation was measured and found to be consistently at 38° C. The capacity values were corrected for physically dissolved oxygen.[11]

The tonometer was coated with silicon† for all the studies reported in Table I. The clean tonometer was rinsed with silicone, distilled water, 30 per cent ammonium hydroxide, and thoroughly with distilled water. It was then dried overnight at room temperature. This procedure was repeated after twenty-five tests.

Oxygen capacities were also determined by the neutral ferricyanide-chamber method which is the Roughton, Darling, and Root[4] modification of Sendroy's[12] technique. The blood was saturated with oxygen in the chamber of the Van Slyke manometric apparatus by mixing with saline solution previously saturated with oxygen. The mixture was then treated with ferricyanide-saponin solution. Corrections for dissolved oxygen were made as described by Roughton and associates.[4]

COMPARISON OF METHODS

Experiment 1.—Oxygen capacities by the neutral ferricyanide-chamber method and the alkaline ferricyanide-tonometer method were compared. Arterial samples were taken from sixteen normal men between the ages of 19 and 63. Oxygen capacities by the two techniques were determined on blood from the same syringe. In order to eliminate any

*Sapoin-Practical, No. P4704, Organic Chemical Division, Eastman Kodak Company, Rochester, N. Y.

†Dri-Film, No. Sc-87, General Electric, Silicone Products, Waterford, N. Y.

influence of time on the results of the oxygen capacity measurements, the alkaline ferricyanide-tonometer method was done first one day, and the neutral ferricyanide-chamber technique first the next day. Tonometers were not coated with silicone. Oxygen contents were determined by the alkaline ferricyanide procedure.

Experiment 2.—The previously mentioned oxygen capacity techniques were further compared by analyzing the sample of blood by the alkaline ferricyanide-tonometer method, using a tonometer coated with silicone. An aliquot of the original sample of blood was again saturated in the same tonometer and analyzed by the neutral ferricyanide-chamber method. The factor of time was controlled as before.

Experiment 3.—This study involved eleven normal men between the ages of 20 and 49 years. Arterial oxygen contents were determined by the neutral ferricyanide and alkaline ferricyanide methods. In each case, blood from the same syringe was analyzed by the two techniques and the factor of time was controlled. Oxygen capacities were then measured by the neutral ferricyanide-tonometer, alkaline ferricyanide-tonometer, and the chamber procedure. The tonometer was siliconed and the factor of time controlled.

TABLE I. COMPARISON OF OXYGEN CONTENT, CAPACITY, AND CALCULATION OF THE PER CENT OXYGEN SATURATION OF HEMOGLOBIN IN NORMAL ARTERIAL BLOOD

NUMBER	AGE	BODY SURFACE (SQ./M.)	OXYGEN CONTENT (VOL. PER CENT) N.F.	A.F.	DIFFERENCE	OXYGEN CAPACITY (VOL. PER CENT) N.F.T.	A.F.T.	C.
1. A. A.	23	2.01	18.89	19.04	±0.15	19.60	19.64	19.65
2. L. W.	29	2.16	17.56	17.98	±0.42	17.75	18.09	17.92
3. H. A.	35	1.84	19.52	19.92	±0.40	19.91	20.17	19.80
4. D. J.	25	1.92	19.60	19.83	±0.23	20.05	20.34	19.92
5. B. S.	49	1.78	16.81	17.10	±0.29	17.21	17.55	17.32
6. T. B.	46	2.14	14.87	15.01	±0.14	15.30	15.55	15.29
7. C. S.	33	1.52	18.95	19.35	±0.40	18.95	19.16	18.96
8. O. S.	20	1.82	18.92	19.12	±0.20	19.04	19.61	19.00
9. A. B.	44	1.81	18.47	18.92	±0.45	19.02	19.09	19.03
10. F. S.	37	1.96	19.31	19.69	±0.38	20.06	19.98	19.87
11. J. T.	32	1.87	19.53	19.87	±0.34	20.07	20.20	20.02
Mean			18.40	18.71		18.82	19.04	18.80
S.D. of Mean			±1.46	±1.50		±1.50	±1.46	±1.45

DIFFERENCE N.F.T.–C.	OXYGEN SATURATION (PER CENT) $\frac{N.F}{N.F.T.} \times 100$	$\frac{N.F.}{A.F.T.} \times 100$	$\frac{N.F.}{C.} \times 100$	$\frac{A.F.}{N.F.T.} \times 100$	$\frac{A.F.}{A.F.T.} \times 100$	$\frac{A.F.}{C.} \times 100$
−0.05	96.4	96.2	96.1	97.1	97.0	96.9
−0.17	98.9	97.1	98.0	101.3	99.4	100.3
+0.11	98.0	96.8	98.6	100.1	98.8	100.6
+0.13	97.8	96.4	98.4	98.9	97.5	99.6
−0.11	97.7	95.8	97.1	99.4	97.4	98.7
+0.01	97.2	95.6	97.3	98.1	96.5	98.2
−0.01	100.0	98.9	99.9	102.1	101.0	102.1
+0.04	99.4	96.5	99.6	100.4	97.5	100.6
−0.01	97.1	96.8	97.1	99.5	99.1	99.4
+0.19	96.3	96.7	97.2	98.2	98.6	99.1
+0.05	97.3	96.7	97.6	99.0	98.4	99.3
	97.8	96.7	97.9	99.5	98.3	99.5
	±1.18	±0.86	±1.14	±1.46	±1.28	±1.38

N.F. = Oxygen content neutral ferricyanide; A.F. = oxygen content alkaline ferricyanide; N.F.T. = oxygen capacity neutral ferricyanide-tonometer; A.F.T. = oxygen capacity alkaline ferricyanide-tonometer; C. = oxygen capacity neutral ferricyanide-chamber methods.

RESULTS

Experiment 1.—Oxygen contents determined by the alkaline ferricyanide-tonometer technique gave a normal arterial oxygen saturation of 98.5 ± 1.41* per cent. The tonometer was not siliconed. Using the neutral ferricyanide-chamber method for oxygen capacities, the oxygen saturation was 99.6 ± 1.31 per cent. The mean difference was significantly different from zero, $t = 6.1936$ and $p < 0.01$.[13] The oxygen capacity values obtained with the alkaline ferricyanide-tonometer method were consistently higher than the neutral ferricyanide-chamber values. The mean difference was significantly different from zero, $t = 6.3641$ and $p < 0.01$. This discrepancy was not due to time. It occurred no matter which method was run first. Saturating the blood-saline solution mixture with air in the chamber for periods of time longer than the usual three minutes did not increase the oxygen capacity values.

Experiment 2.—The possibility was entertained that the previously mentioned differences resulted from factors other than concentration of erythrocytes in the tonometer. In this study, it was coated with silicone to prevent plasma from adhering to its wall. The oxygen capacity of the blood rotated in a tonometer was measured by the neutral ferricyanide-chamber method. The capacity of the same blood was determined by the alkaline ferricyanide-tonometer technique and found to be consistently higher. The mean difference of six tests was significantly different from zero, $t = 4.1555$ and $p < 0.01$. This discrepancy was not due to time. It occurred regardless of which method was run first.

Experiment 3.—Possible error in the alkaline ferricyanide content method was investigated. In eleven normal men (Table I) the mean oxygen content by the neutral ferricyanide technique was 18.40 ± 1.46 volumes per cent and by the alkaline ferricyanide procedure was 18.71 ± 1.50 volumes per cent. The mean difference was significantly different from zero, $t = 9.0938$ and $p < 0.01$. With a siliconed tonometer, the average neutral ferricyanide-tonometer oxygen capacity was 18.82 ± 1.50 volumes per cent, and the neutral ferricyanide-chamber technique gave 18.80 ± 1.45 volumes per cent. The mean difference was not significantly different from zero, $t = 0.5172$ and $p > 0.6$.

The percentage of saturation (Fig. 1) by the neutral ferricyanide content and tonometer methods was 97.8 ± 1.18, with a range of 96.3 to 100.0 per cent. By the neutral ferricyanide content and chamber capacity techniques, it was 97.9 ± 1.14 with a range of 96.1 to 99.9 per cent. The alkaline ferricyanide content and neutral ferricyanide-chamber capacity methods yielded 99.5 ± 1.38 with a range of 96.9 to 102.1 per cent. The alkaline ferricyanide content and neutral ferricyanide-tonometer capacity methods gave 99.5 ± 1.46 with a range of 97.1 to 101.3 per cent oxygen saturation. By the alkaline ferricyanide content and tonometer capacity procedures, the arterial oxygen saturation was 98.3 ± 1.28, with a range of 96.5 to 101.0 per cent; and by the neutral ferricyanide content and alkaline ferricyanide-tonometer capacity methods, it was 96.7 ± 0.86, with a range of 95.6 to 98.9 per cent.

*Standard deviation of the mean.

The mean difference between the saturations obtained using the neutral ferricyanide content and tonometer or chamber capacity techniques was not significantly different from zero, $t = 0.4495$ and $p > 0.6$. The alkaline ferricyanide content and tonometer capacity mean values were similar to the neutral ferricyanide content and capacity figures. Combination of alkaline and neutral ferricyanide content or capacity methods gave erroneously high or low results.

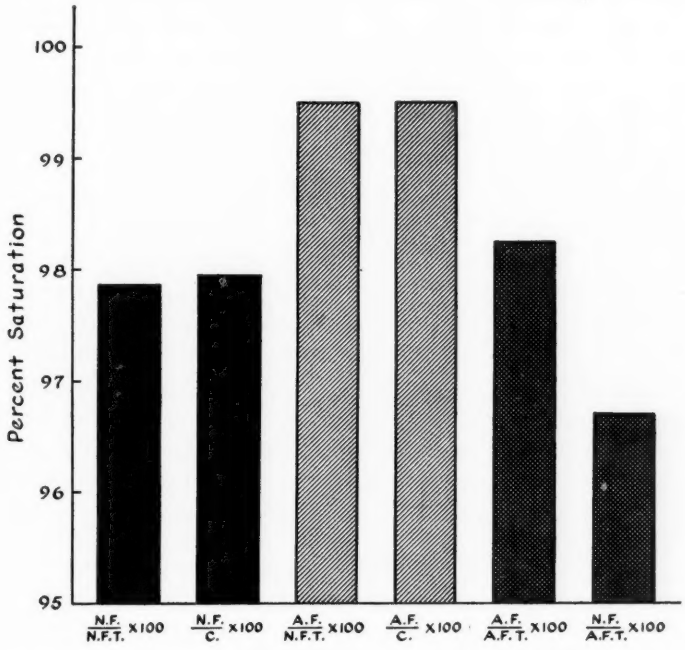

Fig. 1.—Comparison of the percentage of oxygen saturation of normal arterial blood determined by the different techniques of measuring oxygen content and capacity.
N.F. = Oxygen content, volume per cent, neutral ferricyanide method.
A.F. = Oxygen content, volume per cent, alkaline ferricyanide method.
C. = Oxygen capacity, volume per cent, neutral ferricyanide-chamber method.
N.F.T. = Oxygen capacity, volume per cent, neutral ferricyanide-tonometer method.
A.F.T. = Oxygen capacity, volume per cent, alkaline ferricyanide-tonometer method.

DISCUSSION

Arterial oxygen content determined by the neutral ferricyanide method is more accurate than that measured by the alkaline ferricyanide technique. In the first method, carbon dioxide is absorbed by the addition of sodium hydroxide. In the second, the alkaline ferricyanide solution does not absorb the carbon dioxide completely and the oxygen contents and capacities are falsely high. It is important to use saponin with strong hemolytic properties.*

Roughton, Darling, and Root[4] designated three factors as causing a greater oxygen capacity of blood after rotating with air in a tonometer than in vivo. The first was concentration of the red cells because of adherence of plasma to the wall of the tonometer during aeration. This was decreased to a point of no

*The Eastman Kodak Company brand is satisfactory.

significance with silicone. The second was conversion of small amounts of carboxyhemoglobin to oxyhemoglobin. The results of this paper show that when blood was iced, kept in the dark, and analyzed by the chamber method, the values did not differ significantly from those obtained using the neutral ferricyanide-tonometer technique. The third factor was reversion of some inactive to active hemoglobin. Van Slyke[14] found the mean inactive hemoglobin of venous blood, when analyzed immediately after drawing, to be 1.3 ± 0.35 per cent of the total hemoglobin. After standing at room temperature for two hours, this figure fell close to zero. The agreement between the chamber and siliconed tonometer values reported in this study indicates the insignificance of reversion of inactive to active pigment. It is possible that normal arterial blood contains little or no inactive hemoglobin. The chamber capacity method should be used for all oxygen capacity analyses since it is least subject to error. An additional factor is the presence of small physiologic pulmonary shunts which contribute to unsaturation of the arterial blood in the order of magnitude of 1 per cent.[15]

The mean percentage oxygen saturation of the blood obtained by Drabkin and Schmidt[5] using the spectrophotometer was 0.7 to 0.8 per cent higher than the figures reported here. Their range was narrower. They studied only six normal persons, however, and perhaps a larger group would have brought their values closer to the results of this paper. Comroe and Walker's[6] in vivo technique gave results similar to those obtained in the present study. The mean saturation obtained by Wood and Geraci[7, 8] agree with the results of this paper; however, their range of 94.1 to 101.0 per cent is much greater.

SUMMARY

Detailed methods of determining blood oxygen content and capacity were presented. Incomplete absorption of carbon dioxide during the analyses caused contents determined by the alkaline ferricyanide method to be consistently higher than those measured by the neutral ferricyanide technique.

When the tonometer wall is coated with silicone to prevent the adherence of plasma and the concentration of erythrocytes, the oxygen capacity of the hemoglobin measured by the neutral ferricyanide-tonometer technique does not differ significantly from that of the neutral ferricyanide-chamber method. The chamber method is recommended as least subject to error.

Normal arterial oxygen saturation by the neutral ferricyanide content and chamber capacity methods was 97.9 ± 1.14, with a range of 96.1 to 99.9 per cent and by the tonometer capacity technique was 97.8 ± 1.18, with a range of 96.3 to 100.0 per cent.

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THE DETERMINATION OF CREATINE AND CREATININE IN URINE. A CORRECTION FACTOR FOR THE DETERMINATION OF TWENTY-FOUR-HOUR URINARY EXCRETION VALUES

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THE observations recorded here were made in the course of a study which involved the determination of numerous twenty-four-hour urinary steroid excretion values from pregnant individuals. The collections of the urine specimens had to be made by the patients without supervision. It was thought advisable, therefore, to check on the completeness of these twenty-four-hour specimens by the determination of their creatinine content. This method of checking is based on the assumption that the excretion of creatinine shows very little day-to-day variation in a given individual.[1] The Jaffe color reaction[2] for the determination of creatinine has been studied extensively; Clark and Thompson,[3] in particular, have subjected this method to a very thorough investigation. This communication contains additional observations on some problems encountered in determining urinary creatine and creatinine, on the use made of such determinations in judging the completeness of twenty-four-hour urine collections, and in correcting the urinary excretion data derived from incomplete specimens.

MATERIALS AND METHODS

Optical densities were determined in a Coleman Junior spectrophotometer. Maximum optical density of the creatinine picrate solutions using a reagent blank was observed at the 500 mμ setting and this was used throughout. Proportionality between creatinine content and optical density holds up to 3.5 μg per milliliter of solution.

Sodium Hydroxide Solution.—The 2.5N sodium hydroxide may be stored for long periods of time. Since sodium carbonate does not interfere with the formation of the creatinine color, special precautions for the exclusion of carbon dioxide are not necessary.[4]

Standard Creatine and Creatinine.—These were prepared by repeated recrystallization from water.[3] Weyl's test and elementary analysis are not satisfactory means of demonstrating the absence of creatinine in a sample of creatine, since neither method is capable of detecting a contamination of less than 2 per cent creatinine. The Jaffe color test is far more sensitive, but some creatinine color is always found when creatine is subjected to this test in the usual manner. The rate of color development may be determined very easily and the optical density at zero time calculated quite simply.[5] In this way, it is possible to detect 0.1 per cent of creatinine in creatine without special refinements in technique.

Determination of Creatinine.—Coleman cuvettes (25 by 105 mm.) were used. The optical uniformity of the cuvettes should be checked, since some were found to deviate

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from the mean by as much as 1 per cent. One milliliter of a solution containing 20 to 70 μg of creatinine is placed in the cuvette, followed by 4.0 ml. of 1.15 per cent aqueous picric acid solution and 1.0 ml. of 2.5N sodium hydroxide. The solutions may stand for periods varying between fifteen and sixty minutes prior to dilution with 15.0 ml. of water. The readings may then be taken at any time within four hours provided that all tubes of a given batch are read in rapid succession. A set of duplicate standards for two levels of creatinine are included with each batch.

Determination of Creatine.—Creatine is converted into creatinine in 98.5 per cent yield[3] by autoclaving in acid solution, and the creatinine is then determined as above. Clark and Thompson[3] autoclaved their samples in the same tubes of Pyrex glass, which were used subsequently in the colorimeter. Since it is not advisable to do this with Coleman cuvettes, about one-thousandth of the twenty-four-hour urine volume is measured accurately and autoclaved for thirty minutes at 15 to 17 pounds pressure with 4 ml. of the phosphate buffer described below. The solution is made up to 25 ml. and the creatinine content of 1.0 ml. aliquots is determined. In this way, the optical densities almost always came into the linear part of the standard curve. The investigations of Cannan and Shore[6] and Clark and Thompson[3] demonstrated that the optimum acidity for the conversion of creatine into creatinine is near pH = 2; Clark and Thompson, therefore, replaced the mineral acid used previously by a sodium picrate-picric acid buffer at pH = 2. The capacity of this buffer, however, is too low for use in the method given above; moreover, its effectiveness is somewhat precarious since it depends upon the fact that on autoclaving urine for thirty minutes only about one-fifth of the urea present is hydrolyzed. An increase in the time of autoclaving or higher-than-average concentrations of urea cause the pH of the solution to rise, and the conversion of creatine into creatinine becomes incomplete. Such difficulties have been encountered with a few urines, which were either very small or very large in volume. In order to eliminate these uncertainties, a buffer is used, which contains 45 Gm. of potassium dihydrogen phosphate and 58 ml. of 85 per cent phosphoric acid in 1,000 ml. of solution. The phosphoric acid may be replaced by 37 Gm. of borophosphoric acid ($BPO_4 \cdot H_2O$), which is available in powdered form* and which may be measured more accurately and conveniently than the syrupy phosphoric acid. The pH of these buffers should be between 1.7 and 2.0. In cases of doubt, the acidity of the autoclaved sample may be checked. If the pH is above 3.0, the autoclaving should be repeated with a larger quantity of buffer. With urines of average composition, the results obtained using the phosphate buffers and those with the picrate buffer showed satisfactory agreement. The usual amount of buffer does not affect the intensity of the creatinine color, and if larger quantities of buffer have been used, a correspondingly greater amount of sodium hydroxide solution should be added to all tubes of the batch under consideration.

Preservation of Urine Specimens.—If the urine specimen is to be used only for the purpose of checking the completeness of a twenty-four-hour collection, it may be preserved simply by covering it with a layer of n-butanol. Growth of microorganisms destroying creatinine was not observed and creatinine which is converted into creatine on standing[7] will be recovered on autoclaving with acid. If creatine and creatinine are to be determined separately, the specimens have to be kept under conditions which will not permit interconversion of creatine and creatinine. A large number of tests showed that such samples may be kept in the frozen state in containers of polyethylene for at least sixteen months. If containers of glass are used, the time of storage should not exceed three months. At +5° C., the interconversion of creatine and creatinine is very slow, and samples preserved with toluene or butanol may be stored for three months before the interconversion becomes detectable. The presence of large quantities of alcohol seems to prevent the interconversion at +5° C., some samples remaining unchanged for twelve months, but this method is not effective with all urines. It was shown further that conditions which do not permit the conversion of creatinine into creatine also prevent the occurrence of the reverse reaction.

*I wish to thank the Victor Chemical Works for a gift of borophosphoric acid.

Substances Interfering With the Jaffe Color Test.—The Jaffe color test is known to be of a low order of specificity, and widespread occurrence of substances interfering with this test may be anticipated. The n-butanol, "Baker's Analyzed," which was used as the preservative, contains a chromogen which is volatile (butaldehyde ?) and it is expelled together with the butanol on autoclaving. Samples for the determination of creatinine only, which may not be heated, are evaporated to dryness over phosphorus pentoxide in vacuo in order to remove the chromogen. One milliliter of liquid can be evaporated in three to four hours at room temperature, a time interval insufficient to allow appreciable interconversion of creatine and creatinine.[7] At one stage, difficulties were experienced with a chromogen of unknown origin, which was traced to a piece of Tygon tubing. Water which has been in contact with Tygon for a few hours contains a substance which interferes with the determination of creatinine, demonstrating that caution is required when introducing the newer plastic materials into analytical apparatus. Bottles of polyethylene filled with water were allowed to stand for twelve months at room temperature and at +5° C. The water did not contain any substances interfering with the Jaffe color test at any time during this period, in contrast to the properties of Tygon.

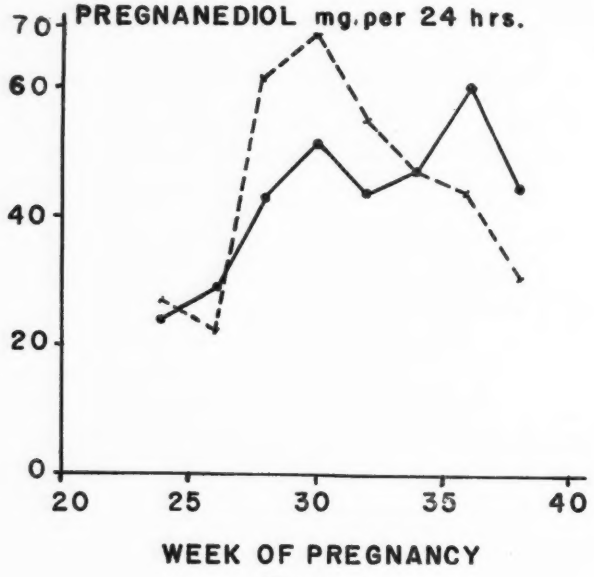

Fig. 1.

RESULTS AND DISCUSSION

The data on the creatinine content of the twenty-four-hour urine specimens collected by our patients were subjected to statistical analysis. The coefficients of variation ranged from 7 per cent to 28 per cent for different individuals. Data in the literature[1] indicate that in a carefully collected series of twenty-four-hour urines the creatinine content would show coefficients of variation of 6 to 10 per cent. Much higher values were found in two out of three individuals studied in our series, indicating incomplete collections of urine specimens. These results demonstrate the necessity of applying corrections when the twenty-four-hour excretion of urinary constituents, such as steroid hormone metabolites, is to be determined under conditions where it is not possible to supervise adequately the collection of speci-

mens. The amount of material found in a particular specimen should be multiplied by the creatinine correction factor. This factor is the ratio

$$\frac{\text{mean 24-hour creatinine excretion of the individual}}{\text{creatinine content of the specimen analyzed}}.$$

The excretion values for pregnanediol obtained during the second half of a normal pregnancy are plotted in Fig. 1. The broken line represents the values as found. The solid line resulted on applying the creatinine correction. The shape of the uncorrected curve is quite uncommon in normal pregnancy; the corrected curve is of the type usually found. In this instance, the coefficient of variation for the creatinine content of the specimens was very large. Usually correction for creatinine content does not result in such a marked change of the curve, but merely tends to bring extremely high or low values into line. In this way meaningful data can be derived from many urine specimens which are grossly incomplete and which would have to be discarded if a means of correction were not available.

SUMMARY

Experimental details are given for carrying out determinations of creatine and creatinine by means of the Jaffe color test.

Methods are described for the preservation of creatine and creatinine in urine samples.

Data from urine collections from individuals without supervision show that creatinine corrections should be applied when quantitative excretion values are to be obtained from such urine specimens.

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KIDNEY CHANGES IN HYPOKALEMIC ALKALOSIS IN THE RAT

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THE relationship between renal changes and the alkalosis which accompanies potassium deficiency has been the subject of recent functional studies. Berliner and associates[10] suggested that the action of the kidney was primary. They felt that depletion of body K upset the normal balance between K and H in the secretory cells of the distal tubules, favoring the exchange of H for Na. Cooke and colleagues[11] presented data illustrating the correction of extracellular alkalosis associated with K deficiency by transfer of electrolyte between the muscle cells and the surrounding fluid. Later, Orloff, Kennedy, and Berliner[12] showed that nephrectomized rats with hypokalemic alkalosis undergo electrolyte transfer when K is given. This suggested that alkalosis was confined to the extracellular compartment, associated with an intracellular acidosis. The problem remains as to why the kidney fails to correct the extracellular alkalosis.

Renal lesions have been reported[1-9] which were both proliferative and degenerative without agreement as to the character or localization of the predominant change. Since structure underlies function, and functional imbalance in renal tubules must accompany this alkalosis, localization and characterization of the pathologic tubular lesion should clarify the problem of pathogenesis.

In order to get the maximum change attributable to hypokalemic alkalosis, several precautions were taken in this study. Protein-depleted rats were used because of the decreased K reserve. An amino acid ration furnished a readily utilized nitrogen source with an extremely low K content. The animals were tube-fed to equalize diet intake so that it was possible to obtain dependable electrolyte balance studies and to avoid negative nitrogen balance.

METHODS

Three-month-old white male Sprague-Dawley rats were depleted to 25 per cent weight loss as described by Cannon and associates.[14] The animals were placed in individual metabolism cages and were fed a synthetic diet in which the nitrogen source was an amino acid mixture.[14] The Osborne-Mendel salt mixture as modified by Hawk and Oser was used. In Experiment 1, eight control animals were fed the diet with the complete Osborne-Mendel salt mixture, and sixteen received the diet with potassium replaced by an equimolar amount of sodium.

Five grams of the diet were mixed with enough water to make a volume of 10 ml. and each animal was tube-fed three times a day at 8 A.M., 3 P.M., and 10 P.M. Full feedings of this mixture could not be started abruptly in these depleted animals or a food shock syndrome developed, causing death. To avoid this, a schedule of gradually increasing feedings were given for the first three days (Fig. 2*A*).

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The water intake and output were measured every twenty-four hours. The pH was measured and the urine was analyzed for glucose, sodium, and potassium. Beginning on the fourth day, two K-deficient animals and one control animal were sacrificed every other day. Blood nonprotein nitrogen, serum total protein, and blood pH or bicarbonate concentrations were measured. The kidneys were weighed. One-half kidney was quickly frozen for enzyme study; one-half was taken for quantitative determination of water, fat, and protein; one-half fixed in cold acetone; and one-half in neutral formalin. Hearts were cut in half in cross section and fixed in cold acetone and formalin. Adrenals were fixed in formalin and the pituitary in Bouin's fluid. The intestine was cleaned of contents, returned to the animal, and the entire carcass was analyzed for water, fat, and protein as described by Benditt and associates.[15] Acid, alkaline phosphatase, and esterase levels in the kidney were determined colorimetrically by method of Gomori.[16, 17] Urine potassium and sodium determinations were made on a Beckman flame photometer. The pH of urine was estimated by nitrazine paper on twenty-four-hour specimens. Nonprotein nitrogens were determined on plasma using trichloracetic acid filtrate, micro Kjeldahl, and steam distillation into boric acid, titrated with hydrochloric acid. Plasma proteins were measured by the densimeter method of Barbour and Hamilton.[18] The pH of blood was measured by a Coleman Model H pH meter using a glass electrode. The blood for bicarbonate was collected under oil and the determination was done by the Van Slyke method.[19] Hemoglobin was determined on a Klett-Somerson photometer. The tissues were embedded in paraffin and stained with hematoxylin and eosin, and by Hotchkiss methods.

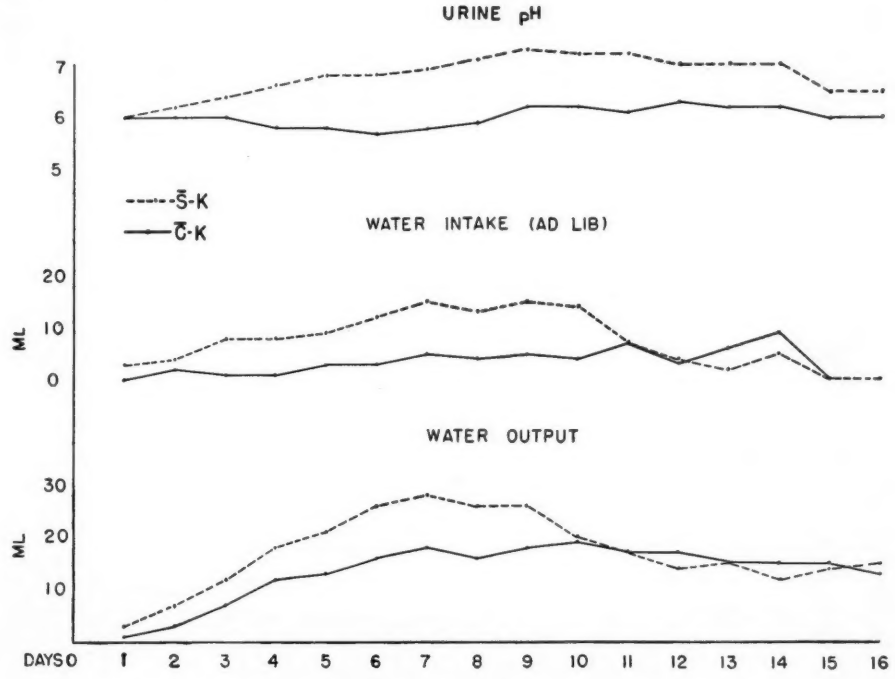

Fig. 1.—Daily averages on all animals.

RESULTS

Experiment 1.—The two groups of animals were comparable during the first four days except for the early and persistent increase in the amount of water consumed and excreted by the low potassium, high sodium animals (Fig. 1) as compared to the controls. There was a gradual change of the urine pH to

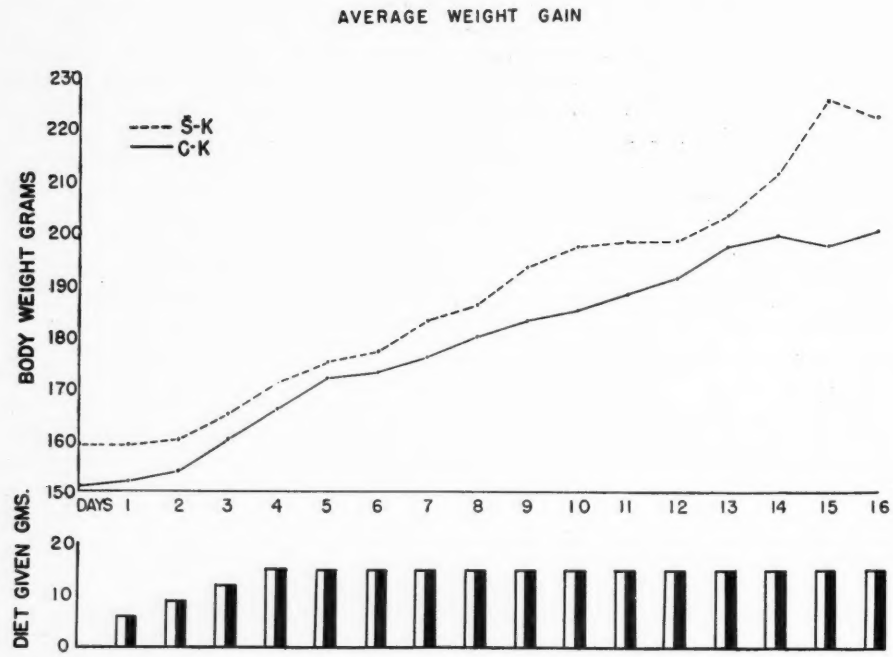

Fig. 2*A*.—The carcass weights paralleled the total body weights closely. Analysis of the carcass for protein, fat, and water showed little variation in the two groups of animals. Experiment 1.

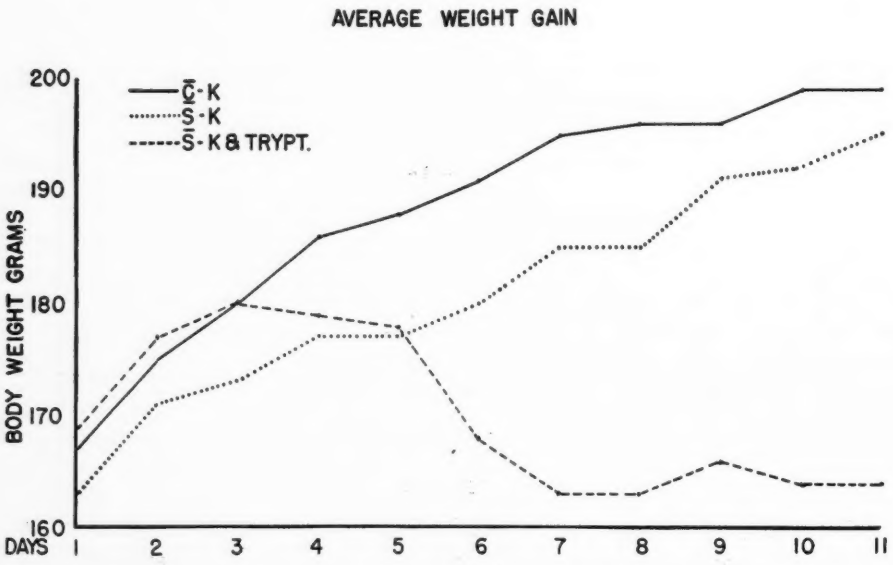

Fig. 2*B*.—The average total body weight charted daily in Experiment 2, shows weight gains closely resembling that of Experiment 1 for the control and without K groups. The third group, in negative-nitrogen balance, lost weight after the first few days.

neutral in the former group (Fig. 1). After the fourth day, the potassium-deficient animals showed less activity than the controls. They began to sit "hunched up" and by the sixth day, several animals had distended abdomens and marked muscular weakness. Some animals had paralysis of the forelegs and all animals became more irritable. There was only one death from ileus, this at seven days. Two animals died on the tenth day, two on the eleventh, and two on the twelfth, apparently from cardiac decompensation.[20] The animals gained weight constantly, the weight curves remaining parallel (Fig. 2*A*). The carcass weights increased comparably. There was an increased retention of feces accompanying the abdominal distention in K-deficient animals but these were removed before the carcass was weighed or analyzed. The carcass analysis done on all animals showed no difference in the percentage of water, fat, or protein between the control and the K-deficient animals. Water balance was comparable although the low K animals consumed and excreted more (Fig. 1). At autopsy there was no fluid in any of the body cavities and no evidence of increased fluid in the tissues.

TABLE I

NUMBER OF DAYS ON K^- FREE DIET	NPN (MG. %)	pH OR HCO_3^- (MM./L.)	BLOOD Hb. (MG./100 ML.)	TOTAL PROTEIN (GM. %)
4	38.3	7.45	10.5	4.4
4	36.2	7.48	9.8	4.8
6	22.7	7.52	10.1	5.8
6	26.3	7.50	10.0	6.2
8	24.9	7.45	13.7	6.6
8	20.6	7.45	14.5	6.9
10	51.1	7.70	13.8	5.6
12	71.0	34.4	14.0	7.3
14	103.0	37.7	12.3	5.7
16	34.1	33.4	14.5	7.4
CONTROLS ON DIET WITH K^-				
4	19.2	7.35	9.9	6.2
6	22.7		10.0	6.3
8	19.5	7.30	11.3	4.9
10	20.6		13.5	6.8
12	21.3	25.0	12.9	6.0
14	18.5	26.8	10.1	4.6
16	22.0		12.9	5.4
16	19.9	28.0	12.9	6.5

Hemoglobin and total protein levels (Table I) showed no change with continued potassium deficiency. The urine sodium and potassium excretion was measured every other day on three control and three K-deficient animals. The sodium excretion fell sharply on the ninth day (Fig. 3). The urine potassium of the test animals fell off sharply to a low level by the fourth day (Fig. 4). This gradually fell to a stable level between the eighth and twelfth days with a slight terminal rise at fourteen days. The control animals maintained a stable output after the full dietary intake had been reached. The water output (Fig. 1) of the test animals dropped after the ninth day and the amounts excreted by the test and the control groups became practically identical. This decrease in the water output of the test animals was accompanied by a decrease in the

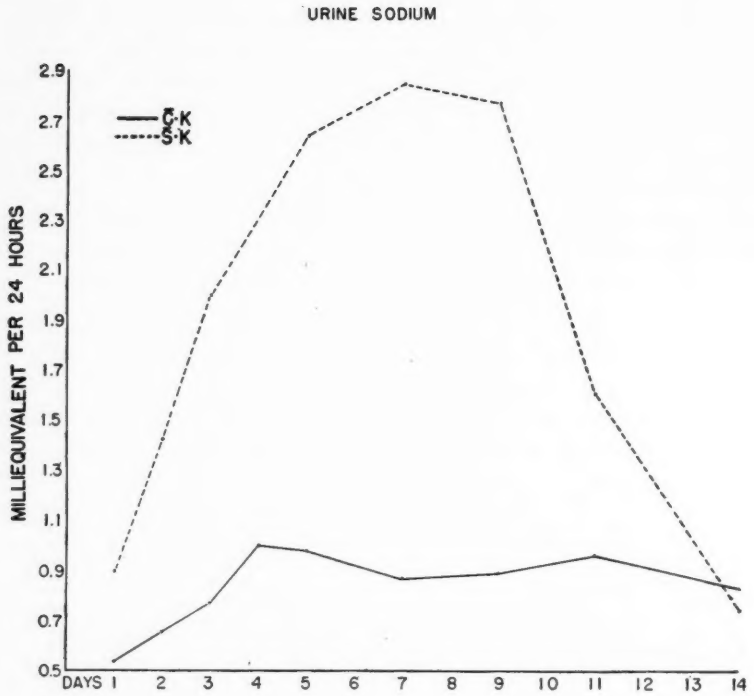

Fig. 3.—The gradual increase in sodium excretion for the first five days is a reflection of increasing amounts of diet as shown in Fig. 2A, but after the ninth day, excretion falls sharply in spite of constant intake.

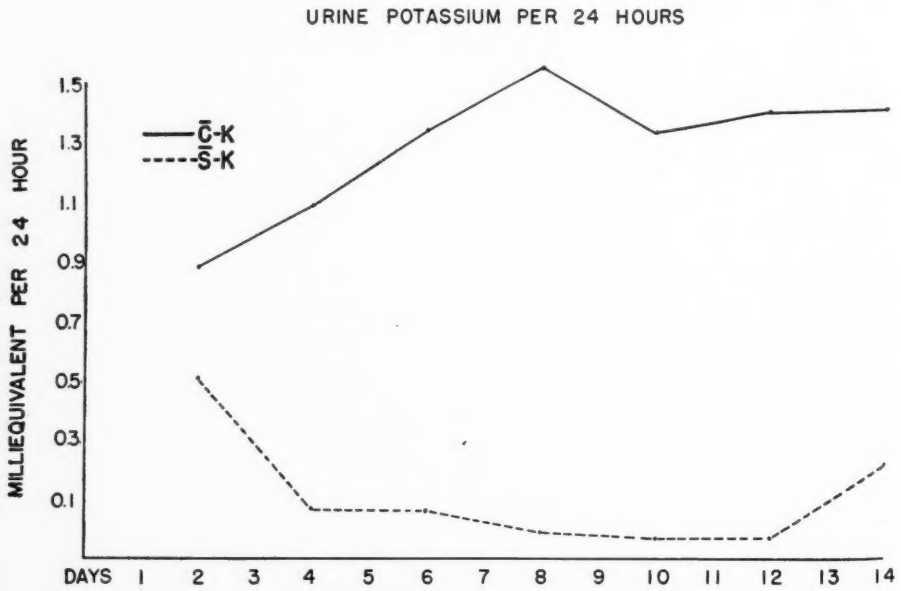

Fig. 4.—Potassium excretion remained low except as the animals became moribund.

ad libitum water consumption. The animals in the test group were in alkalosis on the fourth day when the first pair was sacrificed (Table I). The severity increased with continued potassium depletion. Because of technical difficulties, the HCO_3^- levels of the sera were determined on only the animals sacrificed from the twelfth day on. Nonprotein nitrogen levels of the blood progressively increased (Table I) in the more potassium-deficient animals. This was not true for one animal sacrificed on the sixteenth day, but this animal was the least protein depleted, did not show as marked clinical signs, and the microscopic changes in the kidney were not as severe as the others.

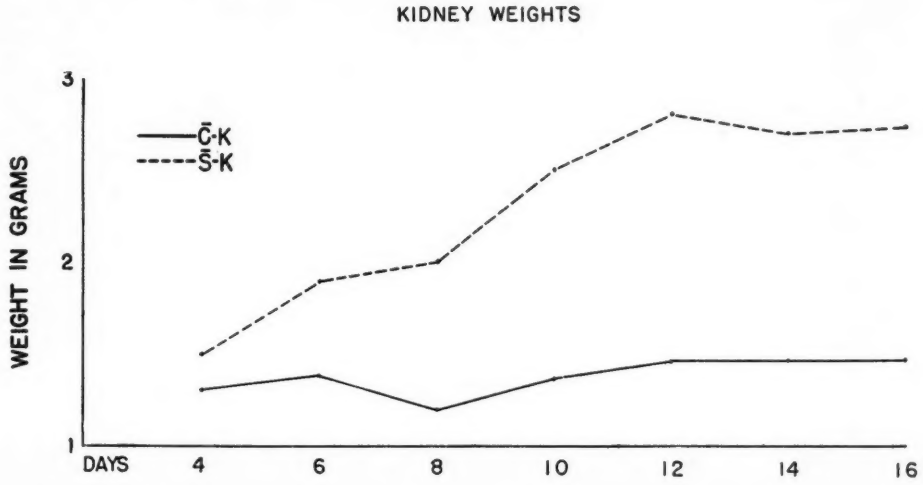

Fig. 5.—Each point for the control animals represents total kidney weight of one rat. Each point for the test animals represents average kidney weight of two animals unless one of the pair had died.

There was a gradual increase in weight of the kidneys in the low K animals (Fig. 5). This increase was not accompanied by a change in the percentage composition of water, fat, or protein components. The kidneys were pale but smooth on gross examination. The first change could be detected microscopically at six days. There was an increased number of mitoses and some binucleated cells in the collecting tubules. There was hydropic change and some dilation of the tubules in the mid-medullary zone. Calcium casts were seen at eight days. These were surrounded by the epithelium of collecting tubules but were still in the lumen. The maximum changes of K deficiency seen at fourteen days (Fig. 7) were characterized as follows: there was an increase in the size of the lumen of the distal and collecting tubules with severe hyaline droplet change in the collecting tubules (Fig. 8). There was an increase in both size and number of the cells in the tubular wall. Some of the tubule cells cytoplasm stained blue with hematoxylin and appeared to be regenerating. There was an increase in the number of mitotic figures, the number of nuclei, and some evidence of amitotic budding. Calcium casts were increased in number. There was moderate hydropic change of the proximal convoluted tubules but no detectable change in the glomeruli. The Hotchkiss

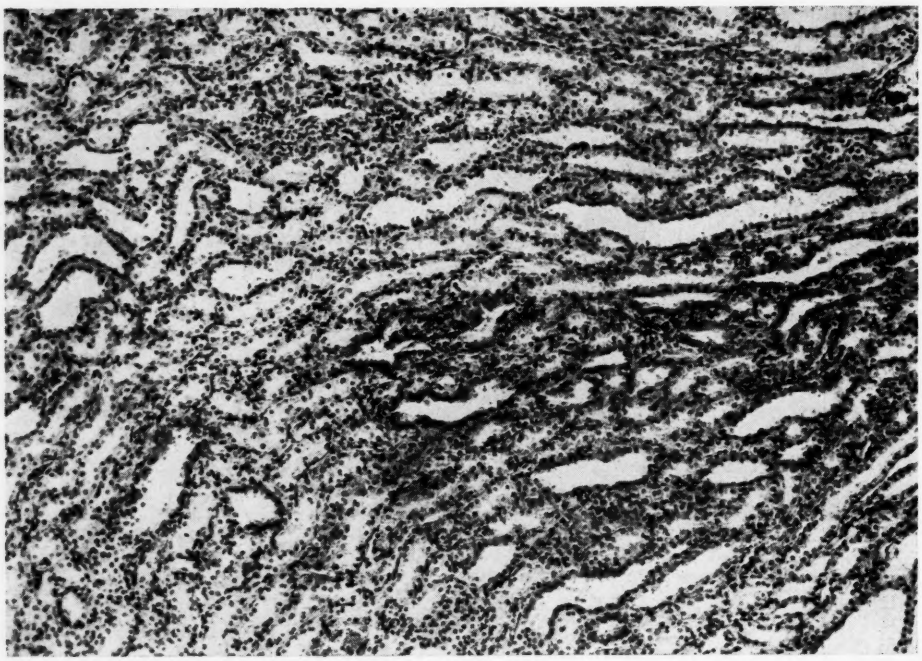

Fig. 6.—Typical kidney changes at eight days showing early tubular proliferative changes. (Hematoxylin and eosin stain. Magnification ×140; reduced ¼.)

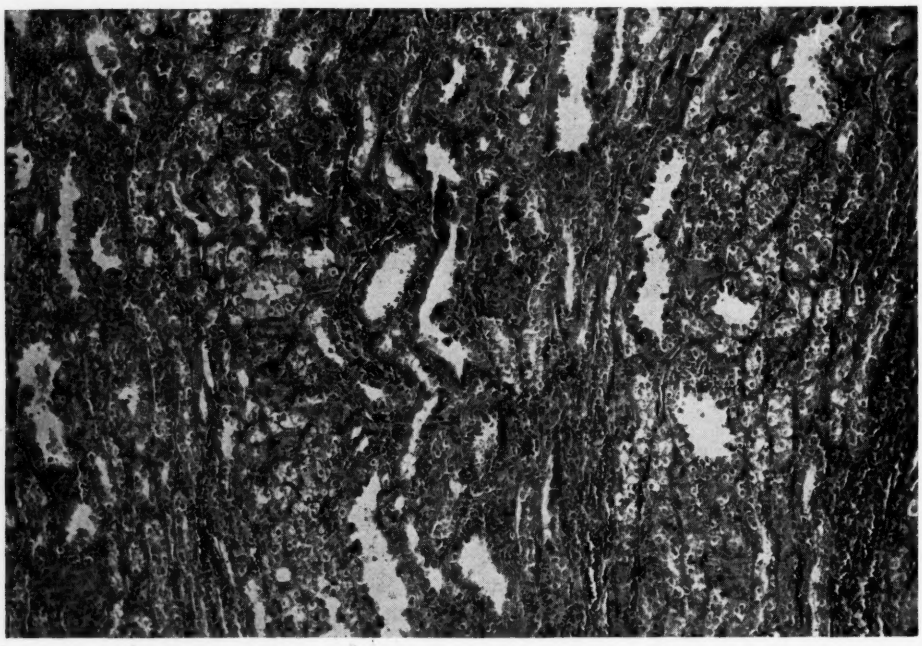

Fig. 7.—Maximum change at fourteen days without potassium. Cells of the tubular wall show increased size, some appear to be regenerating. There is an increase in the number of mitotic figures and the number of nuclei. Hematoxylin and eosin stain. Magnification ×125; reduced ¼.)

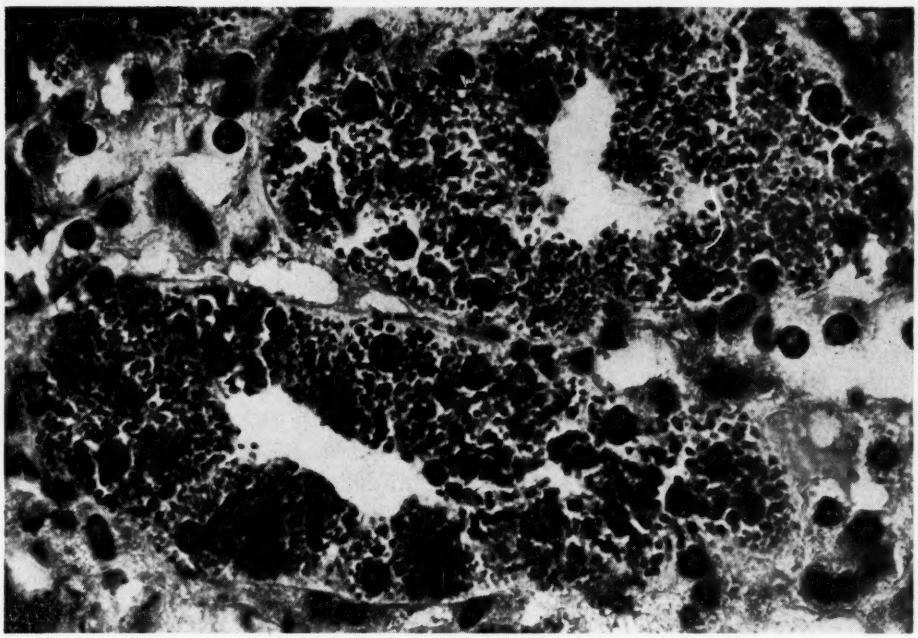

Fig. 8.—Coarse granular changes in the collecting tubules near the papillae, not seen in the control animals. (Hotchkiss stain. Magnification ×900; reduced ¼.)

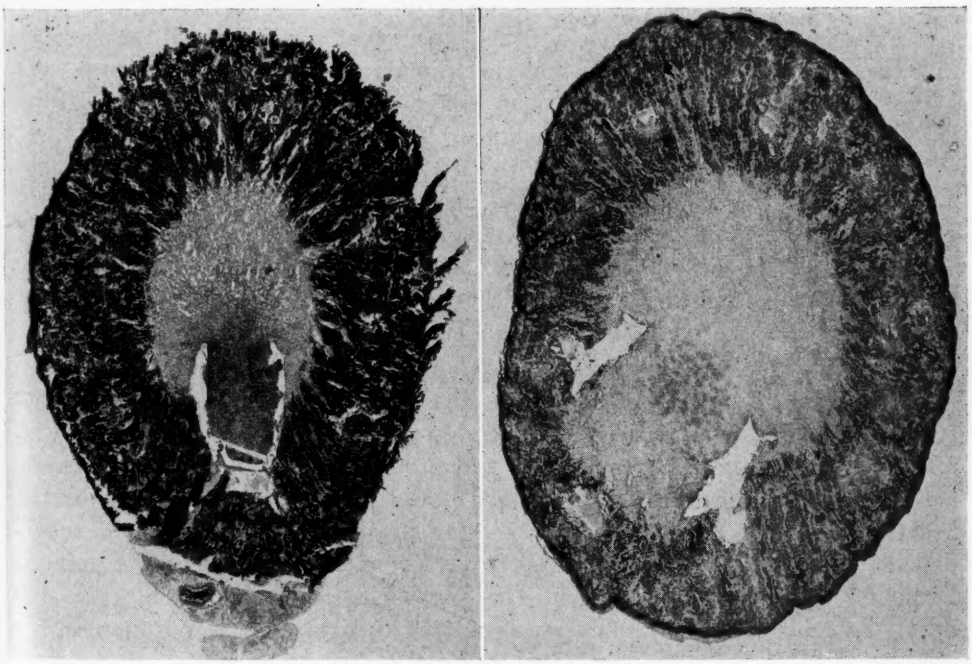

Fig. 9.—This alkaline phosphatase stain emphasizes the localization tubular hypertrophy as well as the general decrease in the enzyme.

stain (Fig. 8) demonstrated coarse granular intracellular substances in collecting tubules near the papillae. These were first seen at twelve days and increased in amount at fourteen and sixteen days. These did not digest with saliva and probably represent a glycoprotein combination, characteristic of hyaline droplet change. Fat stains showed small fat droplets in groups of tubules in the junctional zone. Single tubules showing this change were seen in the subcapsular region at ten days. The twelve-, fourteen-, and sixteen-day animals showed minimal small droplet fatty change in the cortex involving occasional proximal convoluted tubules, though not nearly as extensively as those shown by Follis.[5] Control animals showed a similar change. Enzyme studies showed a decrease in the alkaline phosphatase in K-deficient animals. The control animals averaged 25 to 30 units per gram of kidney while deficient animals averaged 12 to 15 units per gram. Sections stained for alkaline phosphatase emphasized the medullary hypertrophy in K deficient rats (Fig. 9). There was no change in the acid phosphatase or esterase in the kidney.

Heart lesions characteristic of potassium deficiency were found first at six days and were present in every animal from then on. They increased in severity as the animals became more deficient. This is the earliest that lesions have been reported in adult animals on a potassium deficient diet although Darrow[21] reported lesions at four days on a potassium deficient diet plus DOCA. Hotchkiss stains showed no difference in the glycogen content of cardiac muscle of the animals with potassium and without. The adrenals showed no change in size or microscopic appearance. The pituitary was stained with the Gomori trichrome stain and showed no change.

Experiment 2.—The purpose of this experiment was to extend the findings in Experiment 1, which showed that the animals of the K-deficient group were in positive nitrogen balance, and to contrast these animals with a group known to be in negative nitrogen balance. Thirty rats were divided into three equal groups. Group I was given the diet used in the K-deficient animals, and Group II that given the control animals in Experiment I. The third group was given the K-deficient diet except that trypotophan was also omitted. The animals were treated as in Experiment I, except that all were sacrificed on the twelfth day. In addition to carcass analysis, K determinations were made on erythrocytes and muscle (Table II).

The weight gain of the animals in Groups 1 and 2 resembled Experiment 1 (Figs. 2*A* and 2*B*). In contrast to this, the animals without K and tryptophan (Group III) had only a brief weight gain and then a persistent loss (Fig. 2*B*). Carcass analysis for water, fat, and protein revealed no difference in the percentage composition of the animals of the three groups. Lesions of the heart and kidney typical of K lack were present in all animals on the K deficient diet (Group I) as reported in Experiment I. Only half the animals without K and tryptophan (Group III) showed K deficiency lesions at twelve days (Table II). The animals receiving the diet without K but in positive nitrogen balance (Group I) showed uniformly low erythrocyte and skeletal muscle potassium levels. Group III, which was without K and in negative nitrogen balance showed moderate K levels that were less uniform. The

urinary K output was comparable in Groups I and III in spite of the difference in nitrogen balance. The mortality rate in Groups I and III was identical, and there were no deaths in Group II.

TABLE II. CORRELATION OF LESIONS WITH TISSUE POTASSIUM LEVELS

GROUP	ANIMAL NO.	HEART LESIONS	KIDNEY LESION	MUSCLE K^+	RBC K^+
1	Without K^+				
	1	x	x	51.7	77.5
	2	x	x	52.7	80.0
	3	x	x	55.6	77.5
	4	x	x	56.5	85.0
	Mean	100%	100%	54.1	80.0
2	Control				
	1	0	0	89.7	96.2
	2	0	0	90.7	97.5
	3	0	0	91.6	96.2
	4	0	0	89.6	96.2
	Mean	0	0	90.4	96.5
3	Without K^+ or Tryptophan				
	1	0	0	87.5	90.0
	2	x	x	82.0	90.0
	3	x	x	74.8	82.5
	4	0	0	81.8	90.0
	Mean	50%	50%	81.5	88.1

DISCUSSION

The evidence for impaired renal function must be carefully evaluated since hypokalemic alkalosis affects many systems. Freed and Friedman[22] have shown that rats on a low K diet developed a profound hypotension. They observed blood pressures in a range which made renal impairment on a prerenal basis in these animals probable. Tests of renal function in K-deficient rats by others,[23] revealed reduction of the maximum excretory capacity, suggesting a decreased glomerular filtration. There were no morphologic changes in the glomeruli or proximal convoluted tubules of the animals in this study that would account for the observed elevation of the nonprotein nitrogen. Calcium casts were found in the collecting tubules but never in very large numbers. There was no correlation between their time of appearance or number and the degree of renal impairment. How the marked degree of proliferation seen in the distal tubules affected renal function is not known. Detailed measurements using standard renal function tests have not been done in K-deficient animals with renal lesions. These are needed.

The most interesting aspect of the renal lesion observed in this study was that the proliferative far exceeded the degenerative change. After four days, there was an increase in kidney weight (Fig. 5), that progressed to more than 100 per cent by the sixteenth day. This early and continued increase in the kidney size was so apparent at autopsy that it was the most reliable single feature in estimating the relative depletion of a particular animal. Since the diet of these potassium-depleted animals was higher in sodium, one might reason that this increase in weight was due to an increase in water. Analysis of the kidneys for percentage composition of water, fat, and protein revealed

that the K-deficient rats compared closely with the controls. Because these two groups had similar weight gains, correction of the kidney weights for differences in the body size was unnecessary. Durlacher,[4] using rats of comparable size, reported less than half the above increase in kidney weight at the end of the four weeks. This would suggest that the rats in this experiment were more deficient in K than Durlacher's, and emphasizes the value of using protein-depleted rats to study the renal lesion of K deficiency, for these animals had no large reserve of K to utilize. Follis[5] used rats 25 to 28 days old, and although he described cardiac lesions as early as the eighth day, he found increase in the kidney size most marked from the sixth week on. His observations were complicated by the variations in the weight of the animals compared, in spite of paired feeding of test and control groups. Danford,[9] also using growing rats, reported a relative increase in the kidney weight but no significant increase in the absolute weight. This variety of findings, all of the same kind but of widely different magnitude, emphasizes the value of a rigidly controlled experimental regime, in which the maximum change produced is directly attributable to K deficiency. The tube-feeding technique and the amino acid ration employed in this study contributed to a standard set of conditions, which avoided some of the difficulties in previous studies.

The early development of muscular weakness, muscular twitching, apathy, and even paralysis, plus various degrees of ileus, made adequate water and dietary consumption impossible with ad libitum feeding. Wide variations in the sodium content of the diet consumed, a decrease in the amount of water intake, and both the nitrogenous and caloric content of the diet ingested affect renal function and structure. A strict comparison between experimental and control animals is impossible unless these factors are controlled. An example of the result of allowing the experimental K-deficient animals to go into negative nitrogen balance is illustrated by Experiment 2. Here a group of animals was given the diet without K and tryptophan, and a known negative nitrogen balance was created. The early and uniform kidney and heart lesions of the animals in positive nitrogen balance were in sharp contrast to the later and inconsistent lesions of the group in negative nitrogen balance. The latter group apparently utilized their own tissue potassium and probably freed about 1 gram of K for each 10 grams of tissue nitrogen.[24] It seems likely that this enabled them to maintain the K level of their kidneys and hearts (Table II) and gave the delayed and inconsistent experimental findings. In contrast, the animals of Experiment 1 were easily arranged in order of duration of the K deficiency solely on the basis of the extent of the renal lesions.

The increase in the weight of the kidney was not characterized by uniformly distributed hypertrophy and hyperplasia. The change was limited to the distal and collecting tubules (Fig. 9). As would be expected in a short-term proliferation of this degree in a localized area, the microscopic changes were striking. There was sharply increased mitotic activity, an increased number of tubule cells and in spite of an increase in the caliber of the tubule,

there was overlaying of the cells with invagination of tufts into the lumen. There is agreement[3-6] on this element of the lesion. However, most descriptions include extensive degenerative changes[5, 6, 9] which were not found in this experiment. Follis[5] summarized his description of the changes as being similar to the necrotizing nephroses, produced by the salts of mercury or uranium. Henrikson[8] also described these changes as nephrosis. In these previous studies, it is difficult to rule out inanition complicating the electrolyte imbalance as a factor in the production of the degenerative changes. Follis[5] described fatty changes in the convoluted tubules as early as the eighth day, with subsequent necrosis, obstruction of the tubule, and calcification of the obstructing cells. Neither the observations of Kornberg[6] nor those of this study confirmed this degree of fatty change. The presence of calcium casts, similar to those described, have been reported in prolonged alkalosis in dogs without K deficiency.[25] It seems probable that the degenerative changes attributed to K-deficiency were the result of other factors.

The localization of the principal changes in these kidneys was unexpected, for while the distal tubule is known to be active in ion exchange, the collecting tubules have generally been considered passive. The degree of hyperplasia of this area would make it seem otherwise. Ljungberg[26] has shown that the collecting tubules have a concentration of chloride twenty-five times that of the cortex. Microanalysis of serial sections of a punch sample of the kidney, extending from the cortex to the pelvis, showed a progressive increase in the amount of chloride. He reasoned, and the area of hyperplasia in these kidneys appears to confirm this, that reabsorption in the distal system becomes progressively more active. His results indicate that reabsorption was most active in the cells that showed hyperplasia in this study. Further data concerning the electrolyte composition of these cells are not available. This type of data would not only contribute to the understanding of this lesion but would probably solve the puzzling problem of why this kidney maintains the extracellular alkalosis. Cooke[11] suggested that present data would be understandable if the cells of the distal tubules had the same changes in electrolyte composition as the muscle, namely an increase in the Na and H ions. Present analysis of the entire kidney does not reveal this. A study of the electrolyte localization in the distal system of the kidney during K deficiency is now in progress in this laboratory.

SUMMARY

A proliferative lesion localized in the distal and collecting tubules in rats fed an amino acid ration low in K is described. This lesion is uniformly present after the sixth day on the diet and becomes progressively more severe.

Some of the difficulties in evaluating changes due to K deficiency are discussed and the specific effect of negative nitrogen balance is considered.

Evidence for impaired renal function is presented.

The degree of activity in the collecting tubules and its significance are emphasized.

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THE RENAL TUBULAR REABSORPTION OF THIOCYANATE IN NORMAL MAN

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THE details of the mechanism by which the human kidney excretes thiocyanate ion are not clear. It has been reported[1, 2] that the renal clearance of thiocyanate is rather low. This would seem to imply that tubular reabsorption plays a major role in thiocyanate excretion. However, the observation[3-5] that thiocyanate is bound by some constituent of plasma (and therefore not completely filtered at the glomerulus) prevents quantitative assay of the factors influencing its tubular reabsorption merely from data on simultaneous clearances of thiocyanate and inulin. The variability of thiocyanate clearance in the dog has led to the exploration in this species[2] of the possible influences of simultaneous variation of chloride excretion and urine flow. It was found[2] that thiocyanate clearance was highly correlated with chloride excretory rate and much less so with urine flow. In these studies, however, no measurement of glomerular filtration rate was made, and no account was taken of the effect of plasma "binding" upon the thiocyanate ion, so that detailed evaluation of the role of the tubules in thiocyanate excretion was not possible. More definitive knowledge of the mode of excretion of this ion assumes practical clinical importance in the treatment of thiocyanate intoxication. The theoretical importance of thiocyanate reabsorption is that in common with other renal tubular transport phenomena. The present study was therefore undertaken in an attempt to define and evaluate the mechanisms by which the normal human kidney excretes thiocyanate.

METHODS

Experimental Procedure.—Six clinically normal young men in the third decade of life were studied in a series of fourteen experiments consisting of 122 clearance periods. After a suitable priming injection, inulin solution was administered intravenously by a constant speed infusion pump at a rate calculated to produce a constant inulin concentration in the serum. Urine was collected by a multieyed indwelling catheter and the bladder rinsed with a measured volume of sterile distilled water after each emptying. Femoral arterial blood samples were collected at midperiod under oil by means of an indwelling Cournand needle. The subjects were all on known intake of salt and protein for one week prior to each experiment, and were studied in the postabsorptive basal state.

Variation in urine flow was produced by water diuresis or by infusion of hypertonic NaCl and NH_4Cl. Low chloride excretion was secured by employing a low salt diet with a peroral sodium bicarbonate supplement. When it was desired to study the effects of low chloride excretion, the infusion fluids were made up in isotonic mannitol rather than isotonic saline solution. High chloride excretion was promoted by previous ingestion of large amounts of sodium chloride and ammonium chloride and by utilizing concentrated (up to 10 per cent)

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saline solution or ammonium chloride (2 per cent) in the infusion fluids.* In some experiments the thiocyanate concentration in serum was elevated in stepwise fashion by successive injections of NaSCN solution (5 per cent). In other experiments, a single injection was made before starting the observations in order to obtain a relatively constant (albeit slightly decreasing) serum concentration.

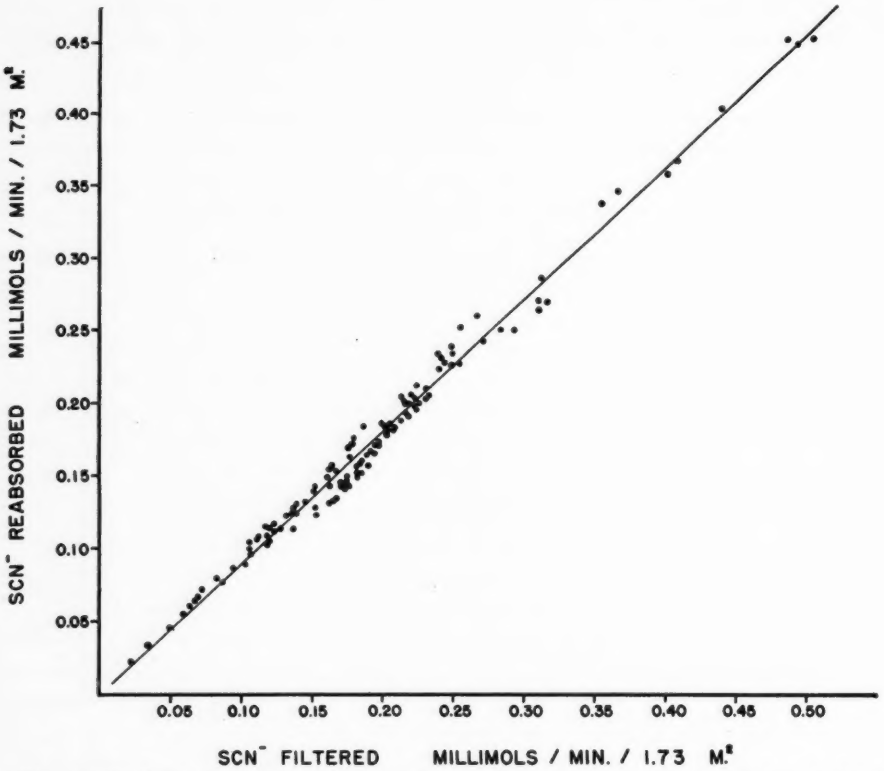

Fig. 1.—Reabsorption rate of thiocyanate as a function of rate of filtration of thiocyanate. Mass plot of data derived from 122 clearance periods on six subjects. Line fitted by method of least squares.

Chemical Methods.—Inulin was determined in cadminum sulfate filtrates of serum and urine by the method of Roe and associates.[6] Chloride was determined in serum by the method of Wilson and Ball,[7] and urine[8] by a modified Volhard-Harvey titration, or in both by iodometric titration.[9] We have found that when the former method is employed for serum, the sum of chloride and thiocyanate are measured as chloride. An appropriate correction was employed. Thiocyanate interfered with neither method when urine was analyzed. Serum ultrafiltrates were secured by adapting the anaerobic pressure dialysis technique of Lavietes[10] to multiple samples by connecting a group of twelve chambers parallel to a common mercury reservoir. Ultrafiltration was performed at room temperature using No. 450 cellophane. Thiocyanate was determined using ferric nitrate reagent[1, 11] in serum, ultrafiltrate, and urine by the technique described by Lavietes and associates[4] except that 10 per cent instead of 20 per cent trichloracetic acid was used.

Calculations.—Glomerular filtration rate was calculated as the inulin clearance, corrected to 1.73 M^2 body surface area. Filtered load of thiocyanate was calculated as the product of the glomerular filtration rate and the thiocyanate concentration in ultrafiltrate. Corrections for the Gibbs-Donnan equilibrium and for plasma water concentration were not made.

*Six clearance periods in one individual have been included despite the fact that he received 1 ml. of mercuxanthine intravenously less than one hour prior to completion of the sixth period. Diuresis did not ensue during the six periods in question, and the results were entirely comparable to the remaining corpus of data.

Excretory rate of chloride and thiocyanate were calculated as the product of the urine flow per 1.73 M^2 and the concentration of the respective ion in the urine. Reabsorption rate was taken as the difference between filtered load and excretory rate. Percentage of reabsorption was calculated by taking the ratio of reabsorption rate to filtered load for each ion and multiplying by 100. Percentage of reabsorption of water was taken to be 100 times the ratio of the filtration rate minus urine flow to the filtration rate. The fitting of curves by the method of least squares and the calculation of partial correlation coefficients and assay of their significance were performed according to standard methods found in most textbooks on statistics.

RESULTS

Data were obtained for 122 simultaneous clearance periods. Urine flows ranged from 1.00 ml. per minute per 1.73 M^2 to 23.2 ml. per minute per 1.73 M^2. Concentration of chloride in serum varied from 92.4 to 128.5 millimols per liter. Concentration of thiocyanate varied from 0.238 millimols per liter to 3.17 millimols per liter in serum ultrafiltrate and 0.250 to 4.22 millimols per liter in serum (1.45 to 24.5 mg. per cent).

Effect of Filtered Thiocyanate Load Upon Thiocyanate Reabsorption Rate.—The results in all subjects in all experiments were sufficiently similar so that the data may be assumed homogeneous and are presented in a mass plot in Fig. 1. The tubular reabsorption rate of thiocyanate was linearly related to filtered load of thiocyanate throughout the entire range studied. This relation was independent of the mode in which filtered load could vary; that is, whether by change in glomerular filtration rate or by change in serum thiocyanate. As the load of filtered thiocyanate increased, there was no tendency for the percentile reabsorption to fall off or to approach a "limiting" value.

Effects of Chloride and Water Reabsorption.—Since thiocyanate reabsorption rate varied linearly with filtered thiocyanate load, it is valid to factor the former by the latter and to utilize this ratio (percentage of thiocyanate reabsorbed) in comparing results obtained at different absolute rates of thiocyanate reabsorption.

The percentage of thiocyanate reabsorbed varied linearly with the percentage of water reabsorbed in all subjects, although with considerably more scatter than in the case of thiocyanate reabsorption as a function of thiocyanate filtered load. This relation is shown in Fig. 2. It was not influenced by chloride reabsorption rate, chloride reabsorption percentage, or chloride excretory rate, and was approximately the same from individual to individual and from experiment to experiment.

The relation of percentage of thiocyanate reabsorbed to percentage of chloride reabsorbed was more complex. The most prominent factor influencing this relationship was the chloride excretion itself. Under conditions favoring high urine chloride concentrations (antidiuresis, high salt intake, or concentrated saline infusion), the percentage of reabsorption of thiocyanate was linearly related to percentage of reabsorption of chloride. Under circumstances favoring low urine chloride concentration (water diuresis or low-salt intake), the percentage of thiocyanate reabsorbed was practically independent of percentage of reabsorption of chloride. These relations are shown in Fig. 3, *A* and *B*, where *A* includes those periods during which urine chloride concentration was high ($\geq$ 90.0 mM. per liter) and *B* includes only those periods during which

urine chloride was low ($\leq$ 9.99 mM. per liter). In *A*, there is an apparent correlation between percentage of thiocyanate reabsorbed and percentage of chloride reabsorbed. In *B*, however, for the same range of percentage of thiocyanate reabsorbed, there is practically no correlation. In those periods where the urine chloride concentration was intermediate between the two extremes discussed previously, the apparent dependence of thiocyanate reabsorption fraction upon chloride reabsorption fraction was also intermediate between those illustrated in *A* and *B* of Fig. 3.

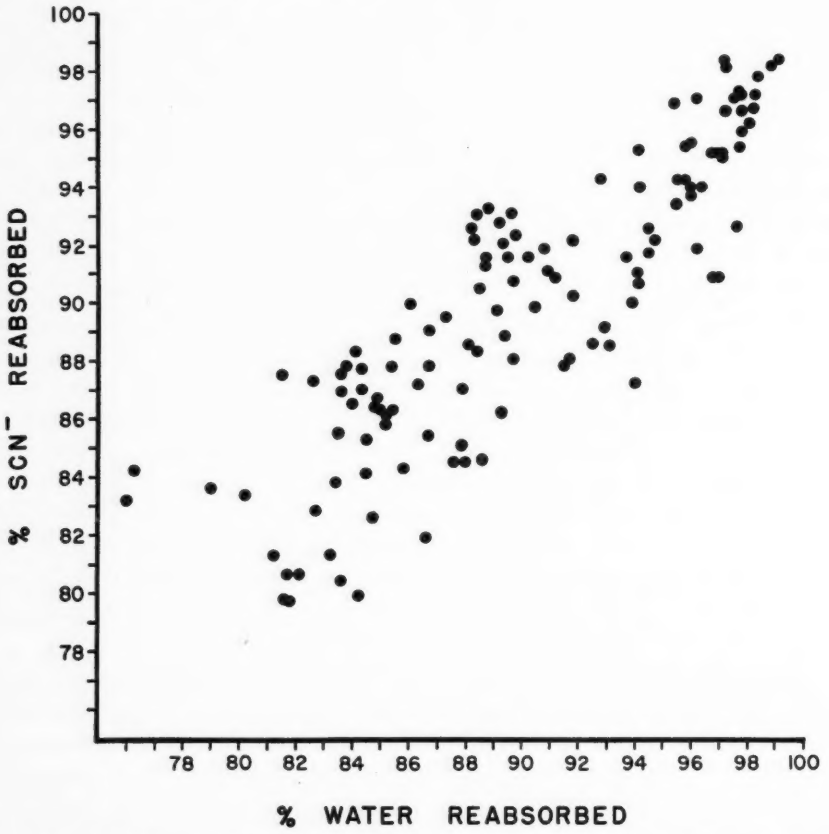

Fig. 2.—Percentage of filtered thiocyanate reabsorbed as a function of percentage of filtered water reabsorbed. Mass plot of data derived from 122 clearance periods on six subjects.

Under each of the previous circumstances the percentage of thiocyanate reabsorbed was related to the percentage of water reabsorbed in approximately the same manner, as shown in Fig. 4. Fig. 4, *A* depicts the same clearance periods shown in Fig. 3, *A*. Both the percentage of thiocyanate reabsorbed and percentage of chloride reabsorbed appear to be linearly related to the percentage of water reabsorbed (and therefore apparently related to each other). Fig. 4, *B* depicts the same clearance periods shown in Fig. 3, *B*. Percentage of thiocyanate reabsorbed bears approximately the same relation to the percentage of water reabsorbed as in Fig. 4, *A*. However, the percentage of chloride re-

absorbed appears almost completely unrelated to percentage of water reabsorbed, the latter varying between 81 per cent and 98 per cent whereas the former remains approximately constant above 98 per cent.

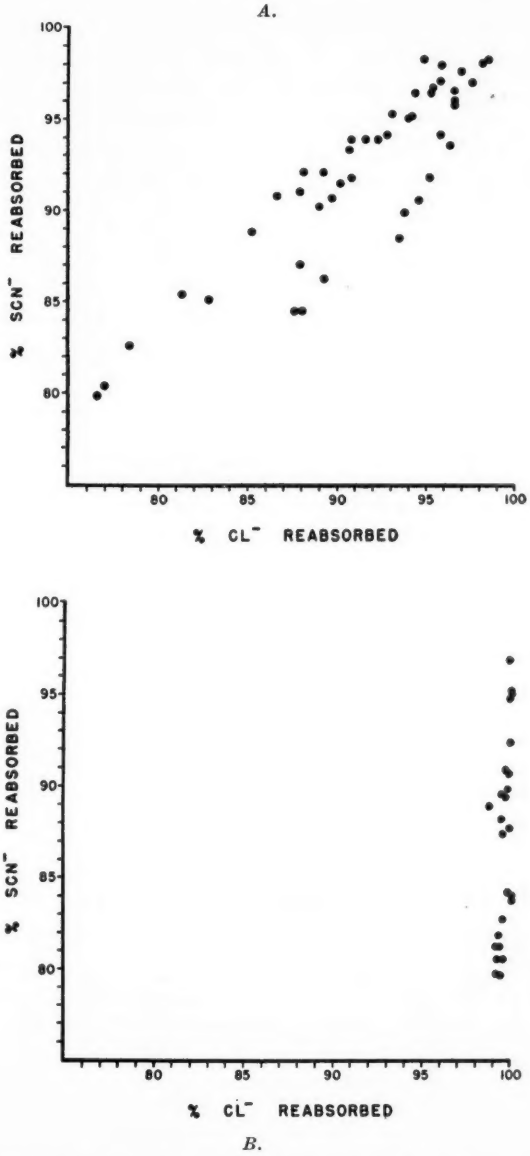

Fig. 3.—Percentage of filtered thiocyanate reabsorbed as a function of percentage reabsorption of filtered chloride. *A* represents forty-five clearance periods (five subjects) during which the urine chloride concentrations were 90 meq. per liter or higher. *B* represents twenty-five clearance periods (three subjects) during which the urine chloride concentrations were less than 10 meq. per liter. Two subjects were common to both groups, contributing nine and ten clearance periods, respectively, to Group A, and ten and seven periods to Group B.

DISCUSSION

Relation of Reabsorption Rate of Thiocyanate to Filtered Load of Thiocyanate.—The linearity evident in Fig. 1 indicates that in the range studied

(which includes not only serum thiocyanate concentrations in the therapeutic range, but also values considerably in excess of those usually encountered in

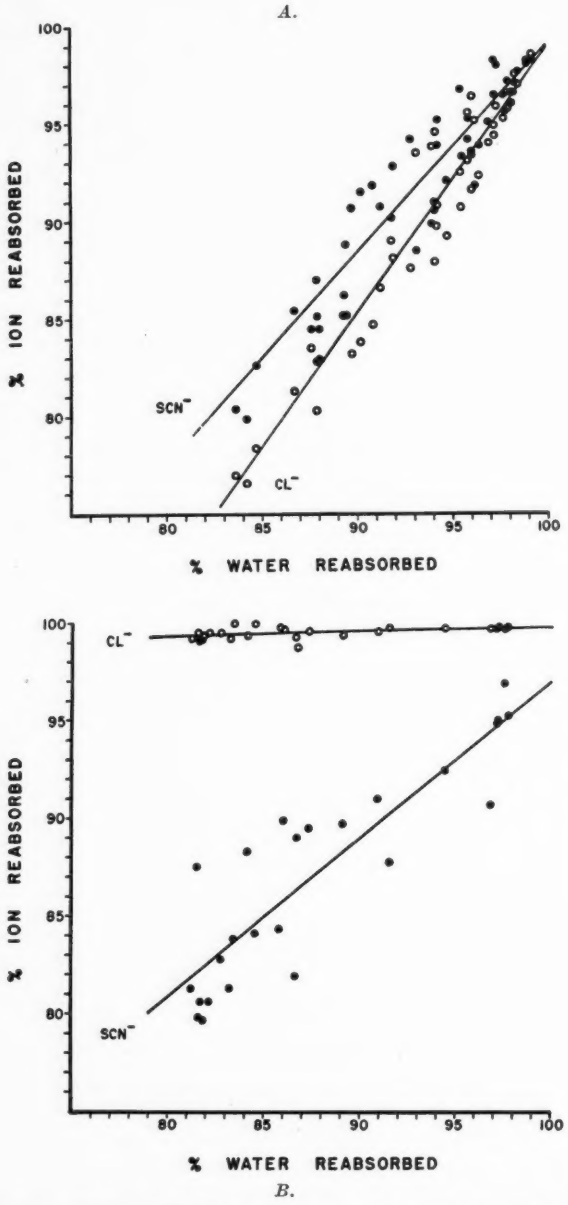

Fig. 4.—Percentage reabsorption of thiocyanate and chloride as functions of the percentage reabsorption of water. The open circles refer to chloride, the closed circles to thiocyanate. *A* represents the same forty-five clearance periods plotted in Fig. 3, *A*. *B* represents the same twenty-five clearance periods plotted in Fig. 3, *B*.

clinical practice) the rate of thiocyanate reabsorption by the tubules bore a constant relation to the filtered load of thiocyanate. By the method of least squares:

$$t_{SCN^-} = 0.898\ L_{SCN^-} - 0.0005$$

where t_{SCN^-} reabsorption rate of thiocyanate in millimols per minute per 1.73 M^2 and L_{SCN^-} = filtered load of thiocyanate in millimols per minute per 1.73 M^2. The intercept is so small as to be of no physiologic significance. This is to say that about 90 per cent of the thiocyanate filtered at the glomeruli undergoes reabsorption. The reabsorptive mechanism shows no tendency toward "saturation" at higher filtered loads. Except for the higher reabsorption fraction, thiocyanate resembles urea in that a constant fraction of the filtered load undergoes reabsorption within the ranges of filtered load that have been studied. The reason why about 10 per cent of filtered thiocyanate should escape reabsorption regardless of the total amount offered to the tubules, is not apparent at present.

It should be pointed out that the close adherence* of the empirical points in Fig. 1 to a straight line is not entirely due to rigidity of natural laws or to unusual experimental and analytic precision. The ordinate represents the difference between a large value (the abscissa) and a small value (the excretory rate of thiocyanate). Since this is so, a close approximation to linearity is not surprising. However, this in no way vitiates the fact of the *linearity itself*. It is merely the *spread* of values around this line that is a fortuitous consequence of algebra. The linearity per se must be regarded as resident in the biologic system itself.

Relation of Reabsorption Fraction of Thiocyanate to Chloride and Water Reabsorption.—Under conditions favoring high urinary chloride concentrations, both thiocyanate reabsorption fraction and chloride reabsorption fraction are related to the water reabsorption fraction (Fig. 4, *A*) and therefore it is not surprising that the thiocyanate and chloride reabsorption fractions appear related to each other (Fig. 3, *A*). However, when the conditions are changed to favor low urinary chloride concentrations (Fig. 4, *B*), although thiocyanate reabsorption fraction still shows a high positive regression on the water reabsorption fraction, the chloride reabsorption fraction varies but slightly with that of water. Under these circumstances, it is not to be expected that thiocyanate should vary with chloride. This is indeed the case (Fig. 3, *B*). The conclusion seems inescapable that the important relation is between reabsorption of thiocyanate and reabsorption of water, and that thiocyanate and chloride reabsorption are related to each other only under such circumstances as obtained during conditions which produce a high urine chloride concentration, when the chloride being excreted obligates the simultaneous excretion of a certain amount of water. This would appear to explain the conclusions reached by Nickerson and Thomas[2] that thiocyanate excretion was related to chloride excretion rather than water excretion. Their experiments were performed during infusion of hypertonic saline solution. Therefore, the conditions under which their observations were made may be considered analogous to those obtaining in our experiments of Fig. 3, *A* and Fig. 4, *A*. Our experimentally deduced conclusion that thiocyanate reabsorption (and hence excretion) is dependent more upon water than upon chloride reabsorption is also supported by partial correlation coefficients as shown in Table I. The partial correlation coefficient between thio-

*Some of the apparent high degree of linearity may represent an interindividual as well as an intraindividual relation, since the six highest values for thiocyanate reabsorption rate were all obtained in a single subject. The remainder of the points, however, seem to be randomly contributed to by all six subjects.

cyanate reabsorption fraction and water reabsorption fraction (the influence of the chloride reabsorption fraction being held constant statistically) is high, and of the same order of magnitude in all groups indicated. The coefficient between chloride and thiocyanate for the entire series is low, although still highly significant. This may reflect other factors having entered into the experiment, or an actual relation between chloride and thiocyanate.* In any case, the influence of water upon thiocyanate is clearly greater than that of chloride. In the group where there was an apparent positive correlation between chloride and thiocyanate (Fig. 3, *A*), partial correlation reveals the actual coefficient to be negative. The reason for this is not readily apparent. Interpreting the statistical data as conservatively as possible, they may be said to support our experimentally deduced conclusions but also to suggest the possibility of a further relation between chloride and thiocyanate.

TABLE I

	PARTIAL CORRELATION COEFFICIENT	
	SCN^- AND Cl^-	SCN^- AND H_2O
High urine Cl^- (45 periods, 5 subjects)	−0.428	+0.784
Low urine Cl^- (25 periods, 3 subjects)	+0.034*	+0.847
All periods: including values for urine Cl^- that were high, intermediate, and low (122 periods, 6 subjects)	+0.265	+0.872

*Partial correlation coefficients between reabsorption percentages of thiocyanate and chlorides (holding water reabsorption percentage constant statistically) and between reabsorption percentages of thiocyanate and water (holding chloride reabsorption percentage constant statistically). All coefficients are highly significant in that their P values are less than 0.01, except for the starred value, which cannot be proved significant.

The therapeutic implications of these conclusions seem obvious. In instances where it is clinically desirable to augment the excretion of thiocyanate (that is, diminish its tubular reabsorption), the most effective way to accomplish this would appear to be induction of copious diuresis. It has been suggested[2] on the basis of dog experiments that thiocyanate intoxication be combatted by administration of large amounts of chloride. While we do not advise against such therapy, we believe that most of the efficacy it may possess stems from its diuretic effect.

Any relationship between dietary halogen content and thiocyanate blood levels, such as has been recently suggested,[2] we believe could be readily explicable on other grounds. It is well known that a high salt intake increases the volume of extracellular fluid. This would increase the effective volume of distribution of thiocyanate (since the latter is essentially confined to this compartment[4, 11]), thereby reducing the concentration of thiocyanate ion. It is also well known that a diet high in salt tends to increase the urine volume since the salt in excess of the needs of the organism is excreted and during its excretion obligates the

*The pitfalls inherent in the application of statistical techniques are almost too well known to require comment. The application of linear partial correlation coefficients in this instance includes as a source of error such possibilities as nonnormality of distributions, nonlinearity of functional relationships, sampling errors, spurious correlations due to common factors entering into calculations of values assumed by each variable, "weighting" due to unequal numbers of periods contributed by each subject, assumptions of homogeneity, and so forth. It is not to be expected, therefore, that under the conditions of these experiments, this technique would yield any other than the grossest indications of the fundamental correlations present.

simultaneous excretion of a certain amount of water. It is not surprising that urine flow tends to vary with the excretory rate of salt.[12, 13] Furthermore, the diuretic response to ingested water is greater in individuals on a high salt intake than when less salt is consumed.[14, 15] Until evidence is produced implicating salt as a primary factor in the excretion of thiocyanate, it seems preferable to explain such interrelations as may exist in terms of known mechanisms.

SUMMARY AND CONCLUSIONS

Thiocyanate ion is reabsorbed by the human renal tubule.

The reabsorption rate of thiocyanate is linearly related to the rate at which it is filtered at the glomeruli, the reabsorptive mechanism showing no tendency to become "saturated" within the range studied. The percentage of filtered thiocyanate undergoing reabsorption varied between 79 and 99 per cent.

The percentage reabsorption of thiocyanate is linearly related to the percentage reabsorption of water, but with a wide scatter.

The excretion of chloride is not an important primary factor in modifying the excretory rate of thiocyanate. The apparent effect of chloride excretion upon thiocyanate excretion observable under certain conditions is explicable in terms of the effect of chloride upon water excretion and of the latter upon thiocyanate excretion.

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LABORATORY METHODS

METABOLISM CAGE FOR SMALL ANIMALS

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DURING the course of investigations of the metabolism of drugs and food additives, it was found necessary to collect separately for an extended period of time the urine and feces of rats. For this purpose a cage which had been used in this laboratory was modified to form an inexpensive and highly efficient metabolism unit.

In general, the operation of this apparatus involved collecting the excreta in a glass funnel equipped with a deflector which directed the droppings to the sides. The feces rolled into a plastic cup arranged in the constriction of the funnel, while the urine was allowed to flow into the stem after passing between the glass wall and the feces cup. (Figs. 1 and 2.)

The essential features of this apparatus consisted of a ¼ inch mesh wire cloth cage, 22 cm. in diameter, which rested on a detachable floor made of ½ inch mesh wire cloth. The floor was supported on the rim of a large glass funnel. A plastic cone deflector of a size sufficient to extend to within 2 cm. of the glass funnel wall was suspended from the center of the cage floor by a wire hook attached to its apex. This cone was made of a circular sheet of celluloid, 0.375 mm. thick with a radius of 9.5 cm., by making a single cut along an entire radius, overlapping the circumference a sufficient amount (about 9 cm.), and fixing permanently in position by means of a few drops of acetone placed between the overlapping sides.

The cup for collecting the feces was made from a circular sheet, 12 cm. in diameter, cut from the same plastic stock used for the deflector. Around the circumference about 15 equidistant radial cuts were made, each about 4 cm. in length. The cup was formed by placing the prepared circle of plastic in the funnel, wedging the center into the constriction of the funnel with a glass rod, arranging the overlapping edges neatly, and finally cementing them together with acetone. The resulting cup was 4.5 cm. deep with a top diameter of 7.5 cm.

In order to provide space for the urine to trickle down the side of the glass funnel without being caught by the cup, the lip of the cup was held approximately 2 mm. away from the sides of the funnel by 12 bits of plastic cemented to the outside of the cup at regular intervals and positioned about 5 cm. below the rim.

The feces cup was held in place by means of a metal spring fastened at one end through a plastic loop cemented to the bottom of the cup, and at the other end, to the funnel stem.

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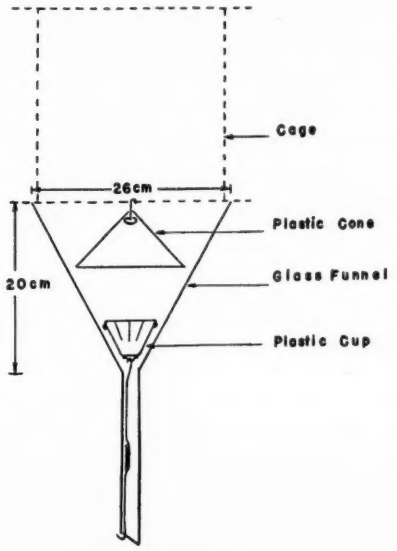

Fig. 1.

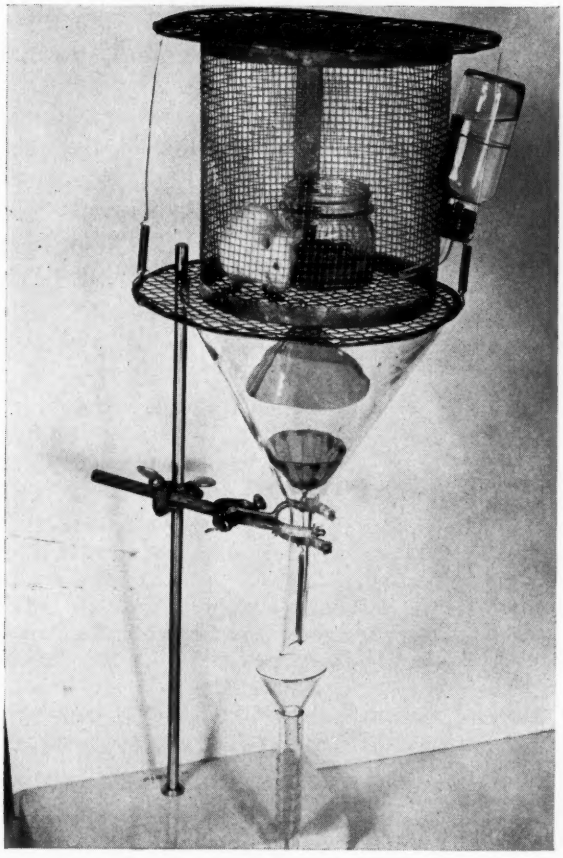

Fig. 2.

The rats were supplied with food in the form of ground laboratory chow in a wide-mouthed, weighted container, and with water previously warmed to room temperature. Since the contamination of normal feces with urine did not occur in this unit, uninterrupted collections could be carried out for several days with no danger of progressive leaching of feces by the urine.

Using this system complete and uncontaminated collections have been made in this laboratory, except in the cases where the feces were excessively soft or liquid.

SUMMARY

A metabolism cage has been described and its construction specifications provided for the collection separately of feces and urine from small animals.

PAPER CHROMATOGRAPHY OF FREE AMINO ACIDS IN BLOOD PLASMA*

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PAPER chromatography of the free amino acids present in blood has presented numerous technical difficulties which have resulted in failure to utilize this technique. Paper partition chromatography has found increasingly widespread use in the chemical analysis of urine and tissue extracts. Blood plasma or serum, however, contains three substances which make the application of identical techniques impossible. These are salts, proteins, and lipid materials. An electrolytic desalting machine has been devised and utilized for removal of the salts.[1-3] It has been shown, however, that there is a loss of some of the amino acids during the process of desalting.[4, 5] In addition, the method is cumbersome. The use of the usual protein-precipitating agents often results in a loss of numerous amino acids.[6] Deproteinization can be effected by ultrafiltration, but this, too, is tedious. The presence of lipid material results in a technically unsatisfactory chromatogram with smearing and smudging.

The most efficient way to achieve separation of the amino acids in blood would be by a series of ion-exchange resins. This procedure is best illustrated by the recent work of Stein and Moore.[7] This technique is extremely tedious and time consuming, however, and does not lend itself to the study of a large number of samples of small volume and concentration.

Recent reports in the French literature[8] have suggested a chemical method of preparing blood for paper chromatography. We have modified such a method and have been able to produce satisfactory chromatograms of human blood plasma or serum. We have summarized our findings in the form of a map of the free amino acid and ninhydrin-reacting compounds which are to be found in normal human blood by our technique.

METHODS

One milliliter of blood plasma is placed in a crystallizing dish measuring 50 by 35 mm. The latter is placed in a vacuum desiccator containing technical grade sulfuric acid and the plasma is allowed to dry overnight.

To the dry plasma, 8 ml. of acetone containing 1.6 ml. of 6 N HCl per 100 ml. is added. The dried plasma is then ground very finely and the crystallizing dish covered with a three by three inch glass plate smeared with stopcock grease. The dish is next agitated for a period of two hours. The acetone solution is then transferred to a 15 ml. centrifuge tube, as much of the precipitated protein being transferred as possible. Five milliliters of the acetone and hydrochloric acid mixture is then added to the residue in the crystallizing

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dish. The agitation is then repeated. The transferred solution in the centrifuge tube is centrifuged for ten minutes. The supernatant is transferred to a test tube. The second batch of the acetone solution in the crystallizing dish is transferred to the original centrifuge tube including again as much precipitate as possible. The second batch is then centrifuged for ten minutes and the supernatant removed and added to the first supernatant. The procedure is repeated a third time so that at the end of the procedure the precipitated proteins are washed three times with the acetone-hydrochloric acid-mixture and the washing is collected in a single test tube.

A special test tube 16 mm. in diameter and 50 mm. in height is placed in a water bath of 37° C. A stream of air dried by passing through a tube of calcium chloride is blown near the top of the tube by a small pipette. The tube is then filled with the acetone extracts of amino acid as evaporation occurs, and evaporation is carried out in successive fractions until the residue is left dry on the bottom of the small test tube. This residue will present a yellow-brown color. It contains the amino acids free of protein and salt but with lipids.

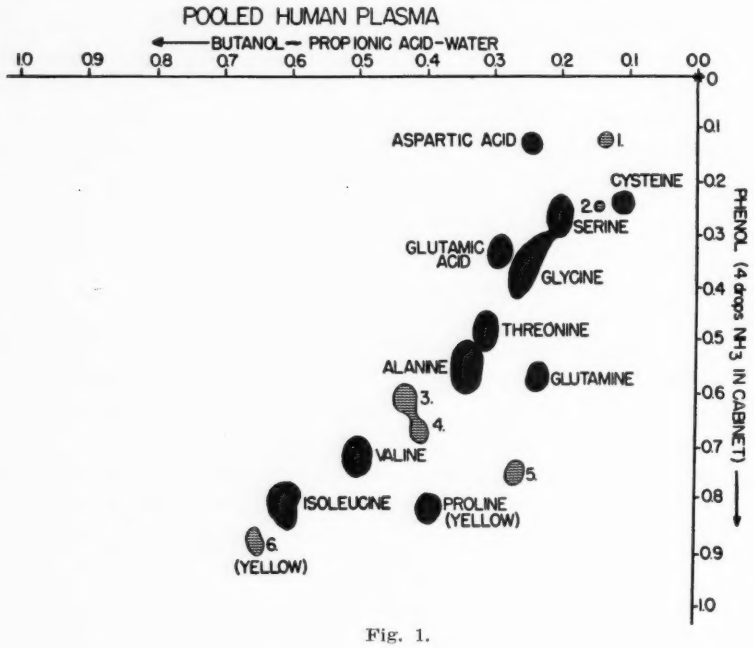

Fig. 1.

Five-tenths milliliter of distilled water is placed in the bottom of the test tube and the residue dissolved. An equal quantity of diethylether is then added and the tube agitated after which the ether layer is removed and this extraction is repeated twice more for a total of three extractions. This procedure removes the lipid materials.

The remaining aqueous solution is then placed once more in the vacuum desiccator over technical grade sulfuric acid and evaporated to dryness.

The dried residue is then dissolved in the desired volume of distilled water, usually 25 to 100 μl, and applied to the filter paper for chromatography.

Chromatography can then be carried out by any of the usual methods. We prefer to use phenol and water as the first solvent and butanol-propionic acid and water as the second solvent. The dried two-dimensional chromatogram is sprayed with an ethanolic solution of 0.1 per cent ninhydrin and the color allowed to develop overnight at room temperature.

Such preparation requires a period of two days. However, a large number of samples can be carried through simultaneously in a simple and uncomplicated fashion.

RESULTS

The previously described analyses were carried out on the blood of numerous members of our laboratory personnel. Both plasma and serum were used, and in some cases pooled samples of plasma were utilized. The chromatograms obtained were quite similar in all cases. Fig. 1 represents a composite map of the free amino acids and positive ninhydrin reacting compounds found in normal human blood by this technique.

In our experience, if the ether extraction is not carried out, the area of the chromatograph proximal to alanine is smudged on the chromatogram and the spots of this area lack sharpness.

Quantitative determination of the alpha amino nitrogen by the Van Slyke method at various stages of the procedure has shown that this technique results in a negligible loss of total alpha amino nitrogen.

Identification of the spots was made by adding a pure amino acid to a sample before running it on the chromatogram and verifying the re-enforcement in color of the particular spot and failure of a new spot to appear.

As can be seen from Fig. 1, eleven ninhydrin-positive compounds have been identified and named on the chart. They are charted as solid black. Six additional spots have not been definitely identified and are therefore shaded and numbered 1 to 6. Spot No. 1 lay in the tail from the cysteine, suggesting that it is a decomposition product of the latter. Other investigators have reported the presence of histidine, arginine, and lysine. These were not found in our material even after spraying the chromatograms with Pauly's diazo reagent. The failure to demonstrate these basic amino acids may be inherent in this method. Spots 2, 3, 4, and 5 remain unidentified. Spot No. 6 has a yellow color and appeared only in the pooled plasma. It never appeared in individual blood samples and therefore may not represent a normal constituent of human plasma. Further speculation as to the nature of these unidentified materials does not seem justified at present.

SUMMARY

A method of preparing blood plasma or serum for paper chromatographic analysis has been described. The free alpha amino acids and ninhydrin-reacting substances found in normal human blood by this method have been described.

We wish to thank Drs. E. D. Churchill and Oliver Cope for providing laboratory facilities and encouragement.

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